

US–Japan trade talks in trouble

The Clinton administration is talking tough on the US trade imbalance with Japan, but should use existing multilateral mechanisms for solving trade disputes if it does not want an historical upheaval in the Far East.

THE annual ritual of negotiations between the United States and Japan on trade is in danger of degenerating into farce. Breaking with the past practice of Japanese prime ministers, Mr Morihiro Hosokawa left Washington at the weekend without having fallen in with the US president's demands — on this occasion, that fixed proportions of Japan's domestic market for telecommunications and automobile equipment should be filled by imports from the United States. The immediate result is that both President Bill Clinton and Hosokawa have been hailed as heroes by their electorates, the former for having been "tough", the latter for saying "NO!". (Another proof that trade negotiations are not a zero-sum game?) But the farce could turn sour if the United States goes on to impose trade sanctions against Japan.

The perennial driving force behind US expectations of Japan is the chronic US deficit in the balance of trade, now running at \$60 billion a year. That is a lot of money, but there is a sense in which it is irrelevant. Since the invention of money and the consequent attenuation of barter, a trade deficit with one country can be arithmetically offset by surpluses with others. For a country to insist that it should be in balance with all its trading partners would imply that it had largely opted out of the international division of labour. In reality, the United States is consistently in surplus with Western Europe, but Washington would rightly resist demands from Brussels that there should be guaranteed shares of the US market for certain categories of imports from Europe. Why should it be surprised that Hosokawa refused precisely such demands?

Truth

The stock reply, of which much will be heard in the United States in the days ahead, is that Japan is consistently a black sheep on trade. For has not Japan enjoyed a trade surplus with the rest of the world for at least the past two decades? The implied argument in that overlooks the simple truth that Japan has made its way in the world by technological cleverness, not only in the design of superior products, but in the technology of manufacturing them for a mass market. There have been two offsetting consequences. The value of the Japanese *yen* against other currencies has risen steadily (making Japanese people individually richer and exports more expensive) and manufacturers elsewhere (including the United States) have set out to emulate the Japanese.

Complaints that Japan's trade surplus derives from unfair practice would be more convincing if they did not so quickly

follow the latest version of the General Agreement on Tariffs and Trade (GATT), agreed in the nick of time only at the end of last year. Clinton's demands of Hosokawa last weekend should properly have been restricted to such evidence as there is that Japan is breaking GATT's new rules. The notion that there should be guaranteed shares of other people's markets for US goods is contrary to the spirit of GATT, and is also unworkable except in dealings with command economies of the kind now fast disappearing. The failure of the notorious semiconductor agreement to win a 20 per cent share of the Japanese market for US manufacturers is proof of that. Plainly, the government in Tokyo is no better able to control what its consumers buy than would be the government in Washington. One misfortune of last weekend's meeting is that it will weaken GATT by declaring that the United States has better ways than the treaty signed last year of looking after its own interests.

Complaints

That is not to suggest that the GATT agreement as it stands is perfection. On the contrary, many of the present agreement's deficiencies were widely advertised in the hectic weeks preceding last December's rushed deal. But one of the complaints levelled at Japan last weekend, that the US company Motorola has not won a fair share of Japan's mobile telephone market, is exactly mirrored by a claim, still not adjudicated, that the British company Cable and Wireless should be allowed to offer such services in the United States. The best remedy for both complaints would be another round of GATT negotiations, not more intransigence at the White House.

The danger with tough talk is that it risks jeopardizing good relations between the United States and Japan at a time when Japan is in an especially delicate condition. It is not merely that the new Hosokawa government is founded on a shaky alliance of reformist parties, but that Japan itself is being forced into a reappraisal of its place in the world. The current recession is one thing. More important is the suspicion that Japan's industrial stature will eventually be overtaken by neighbours in the Far East. Relatively low-cost Korea is already a significant competitor, China a more formidable threat in the long run. Already there are Japanese who would put their money where they believe the future lies. Demands on trade that Hosokawa rightly describes as "arbitrary" could accelerate that tendency. Japan and China will not quickly make common cause, but President Clinton



should perhaps reflect what happens to Japan's trade surplus; until the recession began to bite hard, it was mostly returned (as investment) to the United States, where it helped to finance the federal budget deficit. □

Dust settles on SSC

High-energy physics in the United States did not come to an end with the cancellation of the SSC last year.

THE cancellation last November of the Superconducting Super Collider has been traumatic for the US high-energy physics community. Nobody should be surprised that there is as yet no plan for what will happen next. Hopes that the US administration would apply a poultice to the hurt by promptly opening negotiations for US membership of the European Laboratory for Particle Physics (CERN) at Geneva were always wishful thinking, while the US Congress, at least in the present climate, is not in the business of offering consolation prizes to disappointed supplicants. The cancellation of SSC was, in any case, something of a trauma for the Congress, as the opinions of Texan congressmen will show.

None of that implies that high-energy physics in the United States is dead. For one thing, the US community is probably better supplied with sources of energetic particles, what with Fermilab, SLAC and Cornell, than the rest of the world put together. Then the United States remains the principal source of theoretical work in high-energy physics. It would evidently be a great waste of talent and momentum if these skills were dissipated. The influential High-Energy Physics Advisory Panel (HEPAP) is due in April to produce a strategy for the years ahead. What should it say?

The trick must be to combine a distant objective with the more immediate need to keep the community alive. That means attempting to answer the question of what is likely to be the need for equipment if CERN's Large Hadron Collider (LHC) is actually built, and if it generates the data expected of it — the Higgs boson, the top quark and the like. Then there are several new kinds of particle accelerators that could usefully be built. A machine consisting of opposing electron and positron linear accelerators devised to yield collisions at greater energy than can be had from circular accelerators is just one possibility. But HEPAP should bite the bullet now, and advocate that such a machine should, from the outset, be conceived and built as an international enterprise governed (as CERN is) by an international treaty, preferably open to all who wish to join.

That would not be the equivalent of joining CERN, but of becoming a founder-member of its successor. The US community would be surprised to find that such a decision would readily win invitations to work with the LHC. In the process, it might ruefully reflect that even the SSC might not have been cancelled if its international components had been included from the beginning and not added as afterthoughts. □

Scared of milk

The US Food and Drug Administration warns milk producers that the label "hormone-free" may be illegal.

THE best of intentions notwithstanding, those who in the 1970s did their public duty by calling widespread attention to the powers of recombinant-DNA technology have left a legacy of deep public distrust of anything that smacks of genetic engineering. So it is that the US Food and Drug Administration (FDA) has taken the extraordinary step of warning dairy farmers not to label milk as 'hormone-free' if they cannot certify that it comes from cows that have not been fed bovine somatotropin (BST) to stimulate milk production (see page 585). By adding BST to feed, farmers can increase a cow's milk output by as much as 10 per cent and gain an economic advantage either because they have more milk to sell or they can sell the same amount from a smaller herd.

Taking up what is, for FDA, a combative position, Commissioner David A. Kessler is evidently trying to head off threats of boycotts of milk from BST-treated herds, arguing that there is no known risk from the treatment which FDA approved in November (see *Nature* 366, 192; 1993). FDA offers three arguments against labelling that would imply that BST-free milk is somehow purer or safer than other milk. First, there is no reliable chemical test to distinguish milk from BST-treated and BST-free cows. Second, even ordinary milk contains some natural BST. And third, FDA says it will require anyone labelling milk as hormone-free to keep track of every cow in every dairy herd to verify claims that BST has not been used. Such a requirement would be nearly impossible to meet because of the large numbers of dairy farms that would have to be monitored.

Nevertheless, several large supermarket chains have vowed that they will not sell BST-treated milk for fear of public reaction against a genetically engineered product that anti-biotechnology activists claim is dangerous. But this may soon be a lost cause. The product has been on the US market for only a couple of weeks, but already 10 per cent of dairy farmers along the East coast from Delaware to Virginia have purchased it. In addition, large food processors such as Kraft, which has huge sales of cheese products, and Gerber, which sells baby food, have said they will make no effort to purchase BST-free milk because there is no scientific reason to do so. Products made with milk from BST-treated cows will therefore soon permeate the supermarkets.

The latter-day Luddites now protesting at the prospect of cheaper milk know more about fear-mongering than chemistry. They alone would profit from public hysteria over the use of BST for improving the productivity of US cattle herds. It is to be hoped that FDA's ruling on false advertising will rob them of that prize. Prudently, the European Union has yet to deal with this politically hot issue; its decision on the use of BST has been postponed. □

US and British researchers agree not to seek gene fragment patents

Washington. The National Institutes of Health (NIH) made an eleventh-hour decision last week not to appeal a ruling by the US Patent and Trademark Office (PTO) rejecting its application for patents on some two thousand human gene fragments.

The NIH had been given until midnight last Thursday to lodge an appeal against the decision. It also announced its intention to withdraw a pending patent application on a further 4,448 complementary DNA (cDNA) sequences, in addition to any foreign patent applications.

The day after NIH's decision, the UK Medical Research Council (MRC), which had previously offered to suspend its own patent applications if the NIH acted in a similar way, announced plans to follow suit.

After seeking the advice of government officials, lawyers, representatives from industry and scientists, Harold Varmus, the director of NIH, concluded that pursuing patents on partial or full-length gene se-

quences whose function and practical utility are unknown was neither in the public nor scientific interest, and "seemed like a very poor use of government money".

Rather than promoting the transfer of scientific results out of the laboratory, Varmus said that pursuing the matter further could hamper research collaborations, both in the United States and abroad.

In June 1991, a decision by Bernadine Healy, then the director of NIH, to file for patent rights on 315 human cDNA sequences sparked off a storm of controversy among academic scientists and officials in industry. Two further patent filings were made, bringing the total number of sequences claimed to almost 7,000.

Healy defended her actions by saying that they were needed to protect the commercial interests of NIH while the Patent Office grappled with the issue of whether the patents could in fact be granted. In what was seen as a largely defensive move, Brit-

ain's MRC filed for its own patents, in case the NIH patents were granted.

Last August, the patent office rejected NIH's claims on 2,421 sequences for the second time, and gave NIH six months to appeal.

The sequences were generated using a technique for the rapid sequencing of cDNA developed by Craig Venter while he worked at NIH's National Institute of Neurological Disorders and Stroke. Venter's collection of sequences ranged from non-coding sequences to partial and, in a few cases, full-length coding sequences.

Some of the fragments have the potential to encode protein sequences that can be recognised as being in the same functional class. But Varmus says he does not consider that to be a "functional definition that has practical value — has utility".

The consensus of a meeting of company representatives, government officials, lawyers and scientists held at NIH last December seemed in favour of an appeal. One dissenter from this position was Rebecca Eisenberg, a law professor at the University of Michigan Law School.

Although there appeared to be a consensus at the meeting, Eisenberg says those present all had very different ideas of what they hoped to accomplish by appealing. In a subsequent letter to Varmus, Eisenberg put forward some persuasive arguments against such action, which Varmus says weighed heavily in his final decision. In particular, Eisenberg says she stressed that any decision to come out of an appeal by NIH was unlikely to resolve the matter (as to what is and is not patentable) once and for all.

News of NIH's decision has generated a collective sigh of relief among many academic scientists. C. Thomas Caskey, international president of the Human Genome Organisation (HUGO) and director and investigator at the Institute for Molecular Genetics, Baylor College of Medicine, says Varmus's decision "is wholeheartedly supported by HUGO".

At a recent HUGO summit conference held last month in Houston, Caskey says wide support was given to the notion that the standard for patentability of cDNAs should be "utility based". Unless something can be said about function and utility, patents on even the full-length gene sequence should not be supported, he says.

In his final decision, Varmus seemed to concur with this view. Caskey says that the problem, however, is that US patent law places greater emphasis on chemical composition than utility.

Diane Gershon

Funding cuts favour industry links

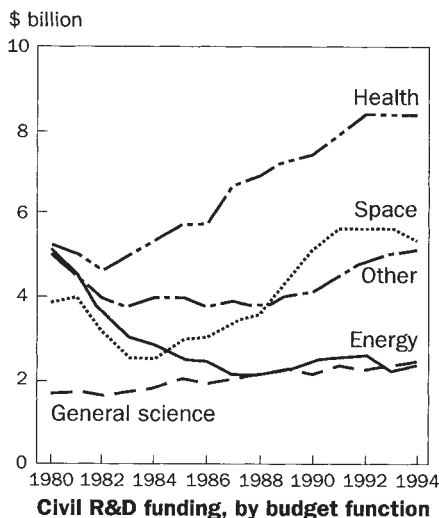
Washington. The rate of growth of US spending on research and development has slowed to an average of only 0.9 per cent a year, one-sixth of that during the period 1975–85, according to a report published last week by the National Science Board.

But *Science and Engineering Indicators 1993* — a biannual survey of US research trends — finds that academic researchers are responding to the more stringent financial climate by forging closer links with industry.

The proportion of academic research funded by the federal government had fallen to 55 per cent last year from 67 per cent in 1980. But funds from the universities themselves and from industry grew to fill the gap, with the latter doubling to 7 per cent.

The United States retains a comfortable lead over its competitors in research and development, with twice as many working scientists and engineers as Japan, and more industrial research and development than all countries of the European Union combined. Its total spending of \$161 billion last year exceeded that of Japan, France and Germany put together, the report says.

But ominously for the longer term from a US point of view, the report also points out that six Asian countries are between them now producing half-a-million science and engineering graduates a year — more than Europe and North America combined.



Following cuts in defence spending, previous shortages of key scientific personnel have given way to a rise in unemployment, which has reached 3 per cent and 3.7 per cent among US scientists and engineers respectively, the report says.

It predicts that surpluses of scientists are more likely than shortages in the coming years. But the growth in wages remains strong, and the number of scientists coming to work permanently in the United States increased dramatically to 23,000 in 1992 — about double the previous annual rate.

Colin Macilwain

Japan faces new questions on HIV in blood...

Tokyo. A renewed controversy over the delays in Japan's introduction of heat-treated blood coagulants, which resulted in thousands of Japanese haemophiliacs being infected with HIV, has been stirred up by a television documentary transmitted by the Japan Broadcasting Corporation (NHK). But the programme has raised more questions than it has provided answers.

As in France, where government officials and scientists have been jailed because of delay in introducing heat-treated products, Japan did not allow the use of coagulants treated with heat to kill HIV and other viruses until 1985, more than two years after their introduction in the United States.

Between 1983 and 1985, there was a boom in Japanese imports of non-heat-treated products from the United States, and many of Japan's 5,000 haemophiliacs were infected as a result. Two groups of haemophiliacs in Tokyo and Osaka are now suing the government and private companies.

The NHK programme revealed strong differences of opinion between officials of the Ministry of Health and Welfare and scientists advising the government on how

to deal with AIDS. In June 1983, a division of the ministry's pharmaceutical affairs bureau set up an AIDS study group headed by Takeshi Abe of Teikyo University to help formulate policy on blood products.

Atsuki Gunji, head of the division, surprised some members of the group when he suggested that the ministry was prepared to circumvent regulations and allow the emergency import of heat-treated products.

But Abe opposed this idea, fearing that doctors would be blamed for any side-effects from the use of heat-treated products that had not undergone clinical trials in Japan. In November 1983, Abe's view won the day and the ministry decided to continue the use of non-heat-treated products while carrying out clinical trials of heat-treated ones, a process that took nearly two years.

The NHK has now shown that a number of incidents in 1983 might have alerted the study group to the immediate threat of AIDS, but for reasons that remain unexplained, no quick action was taken.

For example, 11 days before the study group was established, Gunji was notified by the US company Travenol that it wanted

to recall blood products sent to Japan that were suspected of being contaminated by the blood of an AIDS sufferer; they were returned to the United States in August 1983. A few months later, another US company, Cutter, recalled several thousand bottles of coagulant for the same reason.

Yuichi Shiokawa, of the AIDS study group, when shown a government document describing the recall of the Travenol products by NHK, said it was the first time he had seen it. He said that, if the group had known of Travenol's move, its discussion of emergency imports might have been different.

Gunji, when asked why the study group was not informed, did not give a clear answer but implied that it was not his decision. But his immediate superior at that time, Kazumi Mochinaga, then head of the pharmaceutical affairs bureau, suggested the matter had been Gunji's responsibility.

Another puzzling incident revolves around the first AIDS patient in Japan. In 1983, Abe had a haemophilia patient with typical AIDS symptoms but other members of the study group did not accept Abe's view that the patient had AIDS. The ministry consulted an official of the US Centers for Disease Control who said the patient was undoubtedly an AIDS sufferer.

The patient died in July 1983. But the ministry refused to recognize him as an AIDS sufferer until after it had announced (in March 1985) that Japan's first AIDS patient was a homosexual recently returned from the United States.

A ministry official said that cases of suspected HIV infection in haemophiliacs were not announced earlier to avoid a "big panic" and to ensure a "smooth landing" when the announcement was made. Abe says his hands were tied by his colleagues' decision, and refused to comment on why he did not support immediate changes in the use of coagulants. **David Swinbanks**

... as German executives are charged

Munich. The director and four staff members of a company found to have sold HIV-infected blood products were last week charged with grievous bodily harm, a crime that carries a maximum five-year prison sentence in Germany.

Between 1987 and October 1993, when the company was closed down, UB Plasma, based in Koblenz, supplied hospitals with more than 70,000 litres of plasma that had been inadequately tested for HIV and the hepatitis viruses. The company had tested pooled rather than individual samples of plasma, and had then assessed the results of the colorimetric tests by eye.

As a result, three patients became infected with HIV following transfusions, one of whom has since died from his original illness. A single blood donor was responsible for all three cases.

UB Plasma also sold 43,000 litres of 'industrial plasma', worth DM10 million (US\$5.9 million), to eight different companies between 1988 and 1993. This plasma was used by the companies to make pharmaceutical blood products, and any HIV infection is likely to have been destroyed in standard heat-inactivation processes.

No date for the trial has yet been set, but it will take place in the state of Rheinland-Pfalz, as responsibility for overseeing industrial standards lies with the *Land*, rather than the federal government.

There may be further charges against UB

Plasma. The daily newspaper *Bild* reported on 14 February that in March 1992 the company sold its licence to manufacture and sell pharmaceutical blood products to a small company called Octapharma from Langenfeld for DM100,000.

But it continued to make products and sell them under its own name. The ministry of health in Rheinland-Pfalz took no action, claims the newspaper, even though it had been informed of this illegal action by the Bundesgesundheitsamt, the federal health agency, in July 1992. **Alison Abbott**

... and Le Monde points a finger at the US

Paris. The French newspaper *Le Monde* has claimed that the United States deliberately continued to distribute clotting factors that it knew were contaminated with human immunodeficiency virus to haemophiliacs for economic reasons.

The newspaper last week published extracts of the minutes of a meeting of the Public Health Service (PHS) Task Force on AIDS on 6 May 1985, chaired by James O. Mason — then director of the Centers for Disease Control in Atlanta, and later Deputy Secretary of Health — showing that manufacturers of clotting factors were continuing to distribute products that had not been inactivated by heating whereas heat-inactivated products were available.

The minutes add that the Food and Drug Administration (FDA) intended to put pressure on the manufacturers to stop the practice. *Le Monde* also cites a report to the French government which alleges that transfusion centres in the United States continued to supply unheated products to haemophiliacs already undergoing treatment, and reserved heated products for those beginning treatment, for economical reasons.

The newspaper also quotes Gerald Quinnan from the FDA, as saying that this distinction between groups of patients was made without taking into account whether individual patients were seropositive or seronegative. **Declan Butler**

Spectre of AIDS haunts reports of sick cows

London. Government officials in Britain were last week playing down reports of a 'mystery illness' that has swept through a Cheshire dairy farm and has been linked to a possible infection with bovine immunodeficiency virus BIV.

The illness was reported in the *Independent on Sunday* newspaper as "the first case of bovine AIDS in Britain". The report drew the attention of both local and national newspapers to the plight of farmer Tim Blything, whose 50-strong herd of cows in Kelsall, Cheshire, appear to be suffering from a wide-ranging clinical spectrum including nerve degeneration, weight loss, mouth ulcers and respiratory infections.

So far, two of eight cows have tested positive for antibodies to BIV, by three methods — western blotting, immunofluorescence and ELISA (enzyme-linked immunosorbent assay) — and others have shown positive results on one or two tests.

Despite attempts by government officials to minimize the significance of reports linking the illness to the clinical picture associated with AIDS in humans, local companies are refusing to handle meat from the

herd or to allow milk to be used for pig-feed.

The Ministry of Agriculture, Fisheries and Food (MAFF) is monitoring the herd. But it says that there are no plans to alert other farmers, or to conduct a national sur-



Under threat?

vey of BIV infection in Britain's 11.5 million cattle, as it is not considered to be a notifiable disease, or a threat to human health.

BIV was first isolated in the United States in 1969 from a cow suffering from lymphoma, itself subsequently found to be

due to another virus. But BIV was not known as an immunodeficiency virus until the mid-1980s, when it was found to be one of a group of animal lentiviruses with homology to the human immunodeficiency virus HIV.

Although BIV is not associated with any particular clinical pathology, all the other viruses in this group are serious pathogens that can lead to a progressive and fatal wasting disease in their host animals. According to Joe Brownlie, of the Institute of Animal Health in Compton, Berkshire, whose team is performing tests on the Cheshire herd on behalf of MAFF, these include goats, sheep and cats.

Brownlie says he is keen for MAFF to conduct a national screening survey. "We need to know what the position is," he says.

Andrew Leigh-Brown of the Centre for HIV Research at the University of Edinburgh says that BIV has been around "for thousands if not millions of years". BIV is equally distantly related to other viruses in the ruminant lentivirus group — including caprine arthritis and encephalitis virus, equine infectious anaemia virus, feline immunodeficiency virus — and from HIV, with 25 per cent difference in protein sequence for the reverse transcriptase enzyme common to each virus.

By comparison, the non-human primate retroviruses, simian immunodeficiency virus and African green monkey virus, are more closely related to HIV, with only 11 per cent difference.

Experimentally, BIV has been found to induce only a transient fever and swollen lymph nodes. Natural infection is found worldwide; it has been detected in Australia, New Zealand, the United States and Europe and has not so far prevented human consumption.

Brownlie says he is keeping an open mind on whether the condition affecting the Cheshire herd is due to BIV or another infectious agent, and warns that no study has been conducted for long enough to rule out a true threat to animal health. "I'm unhappy when I hear that all animals infected with BIV do not get disease," he says. "We shouldn't be too dismissive of it all — we just need a proper scientific investigation."

But he says that he does not expect BIV to pose any risk to humans, particularly as it is unable to grow in human cell culture, and has failed to cause seroconversion following two 'needle-stick' injuries in the United States.

Although the precise prevalence of BIV infection in the United Kingdom is not known, it is probably low, according to Ian McConnell, professor of veterinary science at the University of Cambridge. Nor is there any evidence that any of the ruminant lentiviruses cause disease in humans, he says.

Julie Clayton

BST enters battle for consumers

Washington. As the biotechnology industry's first food product — milk from cows treated with bovine somatotropin (BST) — enters the US market, the battle is on between the food industry and genetic engineering activists on both sides of the Atlantic for the trust of US consumers. Two weeks ago, Monsanto Co. began selling the genetically engineered BST after a 90-day moratorium imposed by the US Congress. Activists responded by dumping milk in the streets, defacing dairy billboards, initiating a boycott and asking local school districts not to give children milk from dairies that used the hormone.

Jeremy Rifkin, an opponent of biotechnology, filed a lawsuit against the US Food and Drug Administration (FDA) for approving the drug. Consumers, restaurateurs and dairy farmers have joined the suit, arguing that the agency failed to consider fully the safety of the drug, and should have ordered special labelling on milk products.

Monsanto, which is based in St Louis, Missouri, has been targeting dairies with mail shots, information packs and meetings in dairy regions. Bob Powell, product manager for the drug, trade-named Posilac, said sales have exceeded Monsanto's expectations, and estimates that one-tenth of US dairy cows will be under treatment by the end of the year.

But many dairy cooperatives and milk processors oppose the drug and have told

grocers they will not be supplying milk from cows treated with the hormone. As many as 80 per cent of milk processors in California, the highest-producing dairy state, are said to have either required or asked their suppliers not to use the treatment.

Several have begun printing labels that inform consumers they are not using the drug. The FDA has reacted to the controversy by publishing interim labelling guidelines that allow companies to point out that their milk comes from cows that have not been treated with the hormone — but requires them to provide a context for their remarks, such as giving the reasons why the company had decided against its use.

The FDA says that the hormone is safe for cows and that its presence in milk will not hurt humans. But it is also requiring Monsanto to print labels alerting farmers to increased udder infections and other health problems requiring medication. (Consumer activists fear that such medication may result in antibiotic residue in milk.)

Meanwhile, a survey of UK supermarkets has revealed that many of the major chains would not sell milk from cows treated with BST, even if they were permitted to do so. In member countries of the European Union, the drug is still subject to a moratorium on its use, although this could be challenged in April when the new General Agreement on Tariffs and Trade (GATT) is ratified.

Sally Lehrman

Ireland appoints group to draft science white paper

Munich. The government of the Irish Republic last week announced the members of an independent committee that will carry out the first review of its science and technology policy for more than 30 years. The 18-strong committee, known as the Science, Technology and Innovation Council, is being asked to prepare a draft white paper (policy document) by the end of this year.

The Minister for Commerce and Technology, Seamus Brennan, responded to complaints about the government's handling of research funds last September by announcing an investigation into Ireland's science policy (see *Nature* 365, 197; 1994). But setting up the committee to carry this out has taken



Brennan: listening to advice.

longer than expected, chiefly because of disagreement about its composition.

Despite the delay, the Irish Research Scientists Association (IRSA), the main lobby group for science, has welcomed the announcement. David Fegan, a physics lecturer who acts as the group's secretary, says that the make-up of the committee, which includes six academics, nine industrialists and three civil servants and which is chaired by Dan Tierney, chairman of the Cross Chemical Group, is "better than we'd hoped", as at one point it was uncertain whether the group would include any academics at all.

"The establishment of the committee is a very positive step," he adds, "and we hope that the [draft] white paper will eventually become national policy." If nothing else, he says, it brings the government into direct contact with scientists.

But the problems that precipitated last year's strife are continuing. Forbairt, the newly created agency that administers research grants, has still not been told what its budget will be for this year. It does not yet know if there will be any money at all for new research projects, although spokesman Martin Lyes says he is "hopeful of getting around £750,000".

Meanwhile Forbairt is honouring its commitments to existing projects, for which it will provide about £800,000 in 1994. But it is not risking a second public outcry by announcing a call for new proposals, as it did last year, before funding is secure.

Michael Fahy, from the government's Office of Science and Technology, says that a decision about the Forbairt budget should

be taken within a week. The delay, he says, has been caused by uncertainties over the use of 'structural funds' from the European Commission, which may influence priorities for the office's total budget of £18.6 million (US\$1 million).

Many scientists are angry about the continued absence of grant money, and the general lack of information about what is going on. "We are treated worse than children", says Alan Baird, a lecturer in pharmacology at University College, Dublin, who last month lost a potential postgraduate student to the United Kingdom because he could not offer a definite prospect of a grant.

Meanwhile in response to a threat last autumn from the UK-based Wellcome Trust to withdraw all its research funding from the Republic of Ireland, the government in Dublin has announced an increase of almost 30 per cent in its health research budget.

The government has also said that refunds of valued added tax (VAT) might be allowed on certain items of medical research equipment at the discretion of the finance minister. But Brian Harvey, professor of physiology at University College, Cork, himself a Wellcome grant-holder, says that the concessions may be too small to make a difference.

In a letter to Bertie Ahern, Ireland's finance minister, last November, Bridget Ogilvie, the director of the Wellcome Trust, said that the trust was unhappy both about the fact that it spends twice as much on Irish medical research (around £5 million) as the government, and that it has to pay VAT at 21 per cent on any research equipment that it buys, using money that would otherwise go to research.

Last year, the trust paid £100,000 in VAT. As most items of research equipment cost less than £20,000 individually, the new ruling is likely to make little difference this year. Ireland is the only Organization for Economic Cooperation & Development country to charge VAT on research equipment.

Ahern has so far declined a request to discuss the matter directly with Ogilvie. Nor has he replied to a letter from Wellcome grant-holders in Ireland, sent on 18 January, requesting a meeting to discuss their call for "a realistic response in terms of overall funding policy" to halt the Wellcome threat.

A spokeswoman for Wellcome says that the trust is looking at all options to solve its problems with funding in Ireland, and does not see withdrawal as the only possible course of action. "Our major concern is that Ireland should develop a science policy," she says, "something Wellcome hopes will emerge out of the debate." **Alison Abbott**

Parliament seeks further changes in EU Framework

Paris. The next five-year Framework research programme of the European Union (EU) inched closer to approval last week, when the European Parliament adopted it at a second reading. Under the new procedures of the Maastricht agreement, final approval will require discussion with the Council of Ministers of the parliament's amendments, and agreement on a single text in a 'conciliation committee'.

In December the council of research ministers broke the deadlock over funding of the Fourth Framework programme, scheduled to run from 1994 to 1998, by agreeing to a budget of ECU12 billion (US\$13.6 billion), and a reserve of ECU1 billion. But parliament has now demanded an extra ECU400 million, and also sought guarantees that the agreed reserve will actually materialize.

In particular, the parliament wants to use the extra ECU400 million to increase support for international cooperation and technology transfer, and to juggle the distribution of the budget so as to spend more on socioeconomic research and telematics.

The parliament also wants to reduce the rate at which the council's plans to make the EU's four joint research centres (JRC) more competitive are introduced.

Greece, the current president of the council, is reported to have said that council will probably agree with parliament on these issues. More contentious is likely to be the parliament's demand for access to all information produced by the committees that oversee research programmes. These are made up of national civil servants and commission officials, and meet in secret.

Whatever the outcome, Claude Desama, the president of the parliament's energy, research and technology committee, says that this first experience of the codecision procedures of the Maastricht agreement shows that they need to be changed when the treaty comes up for review in 1996.

Now member states must agree unanimously on a common position, which inevitably reflects the views of the most extreme member state, and leaves little room for negotiations with parliament.

Desama also says that council has not yet entered into the spirit of the codecision-procedure. For example, he says it has refused to try to avert problems by negotiating with the parliament informally.

In a communique, the parliament says that it is "extremely worried, and somewhat astonished" that the council has continued to take decisions "unilaterally" on such matters as the future of the JRCs, and the scheduling of instalments of the Framework budget. **Declan Butler**

Immunologist takes AIDS hot-seat at NIH

Washington. William Paul, an immunologist specializing in the formation of antibodies, will be named this week as the director of the upgraded Office of AIDS Research (OAR) at the US National Institutes of Health (NIH).

Paul, who is 57 years old and has long been head of the immunology laboratory at the National Institute of Allergy and Infectious Diseases (NIAID), will bring strong scientific credentials to the job. But he will also need political skills to handle a sensitive position, created at the behest of Congress under pressure from AIDS

activist groups.

As director of the OAR, Paul will assume control of NIH's \$1.4 billion AIDS research budget. Until now, this control has been exercised by institute directors, such as NIAID's Tony Fauci. The activists claim there is evidence of "a pattern of resistance" at NIH to the new OAR, but Fauci describes this claim as "preposterous".

Paul was selected for the job by Harold Varmus, the director of NIH, who moved swiftly to fill the post after the previous front-runner, Bernard Fields, a Harvard virologist, withdrew for health reasons. "Paul

is a superb scientist who will bring fresh perspectives to the already substantial NIH effort against AIDS," says Varmus. "I am confident that he has the scientific acumen and leadership qualities needed to re-evaluate and shape our approach to AIDS."

Shortly before Paul's acceptance of the post, leading AIDS activists had written to Varmus's boss, Donna Shalala, the Secretary of Health, demanding a meeting to discuss what they described as "a set of problems that threaten to undermine the [Clinton] administration's major AIDS research initiative to date".

In particular, they claimed that opposition to the transfer of control of AIDS research funding from existing NIH institutes to the OAR — a move they claimed was necessary to stimulate new approaches to AIDS research — was threatening to undermine the new office, and deterring the best available candidates from applying for the directorship.

They quoted a letter to Varmus from one leading AIDS researcher (whose name is being withheld), declining an interview for the job and claiming the existence of a "powerful contingent" within NIH vehemently opposed to the revamped OAR. "Unless the political climate improves, it will be difficult to find a director who could survive, much less thrive, in such an environment," the letter said.

Varmus says he has asked for evidence to back up the allegation, and denies the existence of internal opposition to the new OAR. The chief target of the activists' suspicions is Fauci, who has been working as part-time director of the OAR, and whose institute carries out half of all AIDS research at NIH.

But Fauci says that the perception of resistance is "not founded at all" and that the facts speak for themselves. "You are going to see an extraordinary amount of pulling together to make [the OAR] work," says Fauci. "Let's look and see what happens."

The groups whose leaders wrote to Shalala include the National Minority AIDS Council, the Treatment Action Group in New York and the AIDS Action Council in Washington. They were instrumental in persuading Senator Edward Kennedy (Democrat, Massachusetts) and Representative Henry Waxman (Democrat, California) to frame legislation last summer giving the OAR new powers to oversee all AIDS research at NIH.

They are also concerned that the OAR may not receive the \$100 million annual funding that Kennedy and Waxman said it should have — it has been allocated \$10 million in the current financial year — and will fail to take effective control of the NIH AIDS budget during the financial year starting on 1 October, as Congress intended.

Colin MacIlwain

France warned of laboratory 'spies'

Paris. Scientists working for France's national biomedical research organization, INSERM, have been warned of the need to guard against the risks of 'scientific espionage' arising from the increasing internationalization of science and growing interest in its strategic importance.

The warning comes in an article published in the organization's in-house magazine *INSERM Actualité*, by Pierre Louisot, a laboratory director who is also responsible for INSERM's counter-espionage activities.

Louisot says that scientific espionage by both foreign governments and foreign companies is now common in areas such as biotechnology, medical imagery and pharmaceuticals. He also claims that smaller powers keen to obtain the know-how to make biological and chemical weapons are targeting laboratories whose work would not usually be considered as sensitive to national defence.

Louisot says he is committed to scientific openness, but not to what he describes as scientific naivety. He claims that the idealistic urge of researchers to contribute to scientific knowledge is exploited by "more realistic" countries.

Scientists should think twice before making public results in sensitive areas, he says, claiming that more than 90 per cent of scientific espionage amounts simply to organized collection of information that is either published or presented at conferences, but that extra work is then put into filling the "gaps" in this knowledge.

In particular, Louisot claims that researchers are easy prey to questions such as "What are you working on? What areas do you think are worth exploring?" Questionnaires putting such apparently benign questions, he claims, are often a front for organized intelligence gathering.

Increasing international collaboration in biology is also making France more vulnerable to both professional spies and scientists

bribed by foreign countries or companies, claims Louisot. The threat is well controlled within France, he says; but Europe — at least in biology — is a "sieve".

Louisot claims that a scientific counter-espionage policy is needed at the European level. The French counterespionage organization, the Direction de la Surveillance du Territoire (DST) recently launched its own



campaign to promote awareness of industrial espionage and scientific spying; it says that Europe's counterespionage organizations have discussed a joint strategy to protect the ECU8.84 billion Eureka European high-technology research programme.

Louisot claims that INSERM researchers have experienced every sort of espionage, although most of the incidents of which he is aware have been minor. He says that he does not want to create a reds-under-the-bench psychology among scientists, but merely to persuade them to become more vigilant and to take simple precautions — such as not sending confidential information by fax or electronic mail, both of which can be easily intercepted by electronic eavesdroppers.

Declan Butler

Hungary to rationalize its university system

Budapest. A new committee has been set up by the Hungarian government to recommend which of the country's 25 state universities should be allowed to retain their university status, and which should be given the right to award the newly established PhD degrees. The committee was established under a law passed last summer to restructure the Hungarian university system in line with the country's post-communist policies.

The new law requires every full university to have at least two faculties. Many at present have only one speciality, and the committee, made up of academics from universities and research institutes, will recommend which institutions should merge and which should lose their university status. A maximum of eight major universities are likely to emerge from this restructuring.

The law has also introduced for the first time a three-year PhD degree, to be conferred by universities. The new PhD programmes, which began last September, require students to attend formal lecture programmes and prepare a thesis. The committee will determine which departments and faculties will be allowed to participate in the programmes.

The new PhD will eventually take the place of the less structured 'candidate of science' degree, still being conferred by the State Committee of Qualification, a body connected to (but not controlled by)



Due for a merger: Semmelweis Medical University in Budapest.

the Hungarian Academy of Sciences.

While the Candidate of Science degree has been limited to a maximum of 150 students a year, the new PhD programme has already enrolled more than 2,000 students. The Ministry of Education has provided Ft314 million (US\$3.16 million) for the programme this year.

Arpad Csurgay, a professor of informatics at the Technical University in Budapest, admits that the immediate popularity of the new degree is partly a symptom of the poor employment prospects for graduates in Hungary's struggling economy. "It's the best way for young people to postpone the frightening moment of looking for a job when there are so few opportunities," he says.

The higher education law proposes to raise the number of university students in Hungary, which has one of the lowest levels of participation in higher education in Europe, from 100,000 to 150,000 over the next six years. József Hámori, chairman of the University Rectors Conference, hopes that most of the increase will be confined to the non-university higher education sector. At the same time he wants to raise the quality of teaching universities "because we need to increase the quality, not just the quantity, of our students".

Further reforms are expected, and a second higher education law is being drafted by the newly established Council of Higher Education and Science, a body made up of representatives from academic institutions, government and other interested parties, such as industry and local government. A bill based on the committee's conclusions is expected to be submitted to parliament by the end of the year.

This second law will determine the details of the development of higher education over the next decade by, for example, specifying the number of student places to be provided in technical colleges and in universities.

It will also recommend what proportion of the country's gross national product should be allocated to science: the communist state put nearly 2 per cent into research, but the figure has now dropped to 0.8 per cent.

Alison Abbott

Russian newspaper yields to the lure of pseudoscience

Moscow. Serious science journalism in Russia suffered a blow last month when the daily newspaper *Izvestiya* — which has up to now rigorously denounced pseudoscience and refused to give the same prominence as other publications to activities such as parapsychology — published a feature about a tractor mechanic who claims to have invented a time machine.

Almost every news programme on Russian television now ends with an astrological forecast when viewers are advised about their business activities, health care, family matters, sexual behaviour and so on. Millions of Russians, brought up to look on television as the source of state-approved information, appear to be influenced by these instructions.

Parapsychologists can be seen on the screen virtually every day. Even the surprise success of Vladimir Zhirinovskiy in the recent elections has been widely attributed to television appearances on his behalf by Dr Kashpirovskiy, a well-known psychotherapist (and, apparently, hypnotist) who claims that practically all diseases can be cured by

watching his television programmes.

The *Izvestiya* article marks an editorial shift in a newspaper that had previously avoided this trend. It described how Yuri Kunyansky, 50, claims to have overcome the problems of relativity to construct his time machine.

Kunyansky's career has had little connection with solving the problems of space and time; his previous activities include repairing tractors, writing an essay on "resonance in sex" and maintaining machinery at a power station near Moscow.

The report in *Izvestiya* claimed that Kunyansky has been invited by the Russian Academy of Sciences to participate in a seminar headed by Aleksandr Prokhorov, one of the few Russian Nobel prizewinners. It also said that the AGAT company, part of the Russian Space Agency, has agreed to build Kunyansky's machine. Oleg Budakov, a spokesman for AGAT, was quoted as saying that he hoped resources for the machine would be provided by the government not later than 1995.

Despite such signs of support, Kunyansky

is apparently not satisfied with the way things are going. The newspaper quotes him as saying that a properly functioning time machine is still a long way off, as the current design will still take 20 minutes to travel a hundred years in either direction.

But the newspaper itself seems to have had its doubts. In a subsequent issue, it published an article by the same journalist, Sergei Leskov, saying that the Russian Academy considers Kunyansky's theory to be naive, and that nothing should be read into his attendance at the academy's meeting.

Izvestiya has also published a letter from Academician Vitaly Ginzburg criticizing it for deceiving readers about the possibility of constructing time machines.

Ginzburg wrote that President Boris Yeltsin's office, which was reported to have expressed interest in Kunyansky's project, lacks the expertise to judge such matters. He also pointed out that, despite earlier reports, the inventor will not be allowed to present a report at Prokhorov's seminar at the academy.

Carl Levitin

Charities win and lose on hospital mergers

London. British medical research charities fear that they may be asked to foot part of the bill for the surprise decision to make St Thomas's Hospital in London — and not Guy's Hospital — the primary site for a single merged institution.

Under proposals announced last week by Mrs Virginia Bottomley, the health secretary, for the reorganization of London hospitals, acute and specialist services of the two hospitals would be concentrated at St Thomas's, while Guy's would evolve into a "high quality health and academic campus".

But Bottomley did not make any promises that steps would be taken to ensure that clinical researchers at Guy's can move to St Thomas's with the patients who form the basis of their work.

The Imperial Cancer Research Fund (ICRF), which has invested £1.7 million in a new unit to support the charity's research into breast cancer at Guy's, has expressed particular concern over the plans.

"We have asked for reassurances that we will get equivalent facilities at St Thomas's

for our new research programme," says Nick Wright, director of clinical research at the ICRF. "We are waiting for a reply from the minister of health, but if we don't get what we want we will have to ask for our money back."

The unit is to be contained in the United Medical and Dental Schools (UMDS) formed by the merger of the medical schools of both hospitals. It was to have been located in Philip Harris House, a state-of-the-art building originally designed to house clinical researchers from UMDS and patient-care facilities, including day-care and outpatient clinics and an acute mental health-care unit.

Trust funds and charities have pledged to pay about a third of the £142 million cost of the building. But many — including the Special Trustees of Guy's Hospital who have promised around £24 million — are reviewing their donations.

"When the public dips into their pockets to give money to the ICRF they do not expect to be paying for government decisions to merge hospitals," says Wright.

So far, nothing has been decided about the future of Philip Harris House, although one scenario is that the UMDS will take over the top two floors and one third of the other three, while the remaining space will be used to house the primary-care facilities outlined by Bottomley.

She has asked Sir Timothy Chessells, chairman of the London Implementation Group, to convene a working party to look at the costs of reorganizing facilities at the

two sites.

Lynn Carlisle, head of research services at UMDS, said it would be like "one of those Chinese word games" with a lot of shuffling of research groups between the different sites. "There is the potential for building something quite special in the long term," she says. "However, most of the thousand or so researchers here will be affected, and we are going to have a couple of years of significant disruption."

Bottomley had more surprises up her sleeve when she announced that the Royal Marsden Hospital in west London would be granted trust status. The hospital, which has close links with the Institute of Cancer Research, had been recommended for closure by government-appointed review bodies last June.

The Cancer Research Campaign (CRC), which provides about £10 million a year to fund research in the Chester Beatty Laboratories at the institute, announced that it was "very happy" to be able to shelve contingency plans that had been made to move research out to the hospital's other site, about 12 miles away at Sutton in Surrey.

No decision has been reached on the future of the Charing Cross and Hammer-smith hospitals. These have applied for joint trust status, although the precise arrangements will depend on the outcome of a study being undertaken by Sir David Phillips, former chairman of the Advisory Board for the Research Councils, and Sir Rex Richards, a former vice-chancellor of the University of Oxford.

Fiona Gammie

Accounting rules meet with relief

London. University staff in Britain say they have been relieved by the details of plans published last week to introduce greater accountability into the way in which universities and other higher education institutes make use of government grants.

The proposals have been published by the Higher Education Funding Council of England (HEFCE), and outline how universities will be expected to provide the council with an annual breakdown of the allocation of their block grant to departments and faculties from July 1995.

But universities will not be asked for a detailed breakdown of factors such as staff costs and project grants. The aim is "not to impose undue administrative costs on institutions", says Roger Grinyer, an information officer for the HEFCE.

Paul Cottrell, assistant general secretary of the Association of University Teachers, says that the union recognizes that the government has a right to know how its grants are being spent, but had been concerned at the prospect of a mechanism that would allow politicians to veto particular projects. "The only thing that worries us now is the hint that if the internal allocation does not tie in with government priorities, then action could be taken," says Cottrell.

But any such sinister motives are denied by the HEFCE, which explains that the paragraph in question is intended to ensure that future grants go where they are most needed. □

China and Taiwan build science links

Tokyo & Beijing. The exchange of scientists between Taiwan and mainland China is beginning to grow rapidly, despite severe government restrictions on links between the long-estranged neighbours.

Earlier this month, the topic of scientific and technological exchanges featured on the agenda of semi-official talks in Beijing between Taiwan and China, reflecting the growing links in this area.

It was the second such meeting following a ground-breaking summit in Singapore last April, although it still highlighted the huge political gulf that still exists between the two rival Chinese governments, and little substantial agreement emerged.

Since 1989, Taiwan's National Science Council (NSC), a leading government body for funding research, has supported a small but growing programme to encourage links with the mainland. It started with sponsorship of scientists from Taiwan to attend international conferences in China, and now includes funds for short-term visits of a few

months by scientists from either side.

The programme has sponsored a number of cross-strait science and technology conferences. Almost US\$1 million has been invested in the programme by the Taiwanese government, and about 300 scientists from both sides have been supported since its start.

In January, a 25-member mainland delegation headed by Zhou Guang-Zhao, president of the Chinese Academy of Sciences, attended a cross-strait conference on steel and material sciences in Taiwan. The delegation later reported that it had been struck by the enthusiasm on both sides to promote cooperation in science and technology.

But there are still many government restrictions hindering such exchange, particularly on Taiwan's side. There are no direct flights between the two countries and last month's delegation had to travel via Hong Kong. Similarly there are only indirect postal, telephone, and fax links with the mainland.

David Swinbanks and You Qin Li

Impediments to Italian science

SIR — Alison Abbott, writing about Italian academic promotion, has recently reported (*Nature* 366, 293; 1993) that a competition for professor in haematology (*concorso*) has been declared invalid by the court because the committee charged with selecting the winners “failed to distinguish the precise contribution made by candidates to collaborative papers whose authors also included a member of the committee”. Abbott states that this is the first time a decision of a particular committee has been declared completely invalid in Italy.

However, in April 1993, the *concorso* for 24 professorships in paediatrics was declared invalid by the *corte dei conti* (court for administrative control) because the committee failed to apply the rules in selecting the winners. Both the haematology and the paediatric *concorsi* were denounced in *Nature* (353, 10; 1991 & 356, 556; 1992) and in the *Lancet* (2, 1337; 1992) because several of the losers had significantly better scientific credentials (based on indices of the impact of their publications) than the winners.

Thus the role played by the criticism of leading scientific journals cannot be underestimated, having been instrumental in changing the ‘old’ system. The discussions generated by *concorsi* have prompted the Italian Minister for Universities and Scientific Research to propose a change in the promotion system.

In this proposal, the committee will select a number of winners exceeding the number of available positions. Subsequently, each university with an opening will appoint a professor chosen from the winners. The idea is that the committees, being able to select more winners than before, will include candidates with the best scientific credentials in their list of winners. However, this may easily result in their exclusion from professorship. In fact, universities with openings will be left free to appoint anyone from the winners list. Thus, in some universities (described in a book by R. Simone, see *Nature* 366, 642; 1993), under the manipulating influence of local academic élites, it may happen that the best candidates, even if included among the winners, will not be appointed by any university and will lose their positions. The problem can be solved only by selecting and enforcing strict criteria for evaluating scientific credentials (publications in the international literature and their quality based on citation indexes), rather than leaving the committees free to establish criteria that may equate publication in local Italian journals without peer review with publication in leading international journals.

The core of the problem is that Italian universities lack mechanisms for promot-

ing their scientific productivity and this is reflected in the lack of incentives to get the best professors. Thus universities may be happier to extend their favours to the local *baroni* and appoint a loyal pupil rather than selecting a brilliant scientist who may highlight the surrounding mediocrity. The traditional monopoly on power will be reinforced rather than challenged by the proposed changes. Members of the committees may now claim that they did not exclude the best candidates from the recommended appointment list.

While the Italian university system is succumbing to apoptosis, parliament, instead of introducing valid criteria for academic promotion, has just postponed the age of retirement of full professors from 70 to 72 years. Is this the new wave everybody is waiting for?

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SIR — Italy’s presence at the European Molecular Biology Laboratory (EMBL) is disproportionately small compared to its financial contribution and, understandably, Italians resent this situation. But we do not believe that EMBL is acting unfairly, nor that international organizations should necessarily count heads on the basis of nationality (see *Nature* 367, 205; 1994).

As Italian scientists who have worked abroad as well as in Italy, we should like to comment on the general situation of Italian scientists and job opportunities. In our opinion, the small numbers of Italian scientists in foreign countries cannot be explained without taking into account the Italian academic system.

The level of scientific education in Italy is lower than that of several other European countries. The Italian title ‘*Dottore*’ is given at the end of an undergraduate programme, the equivalent of a master’s degree. Italian universities have recently introduced PhD programmes, but the relative lack of competitive research makes it difficult to train graduate students.

Italian students can be trained in foreign PhD programmes, and continue their training abroad. But, as previous correspondents have pointed out, a long stay abroad and the consequent better training are not recognized by the Italian academic system, which has close links with political interests. An “international” *curriculum vitae* is not better than an Italian one; it is simply different and does not fit the academic requirements of the Italian system.

Consequently, young scientists are discouraged from spending long periods

abroad, as it is far more useful to nurture political friendship with appropriate members of committees that control the *concorso*. Ultimately, Italian scientists have to decide whether to remain in a foreign country or try to return home, where they can make little use of qualifications acquired abroad. This is why Italy experiences an otherwise inexplicable brain-drain, given the fact that it has the money, the culture and the people to build a serious scientific establishment. On the other hand, Italy has not developed a biotechnology industry, so there is virtually no job market for biologists and biochemists. This creates a further gap between Italy and its European partners.

In conclusion, it is not surprising that the Italian scientific community is not flourishing at EMBL. Italian scientists are poorly qualified to succeed in such an environment. Moreover, they do not feel it is important to stay abroad to learn new methods for their research, since in many cases such techniques will be impossible to reproduce in Italian laboratories. Nor do they feel compelled to publish good papers, for the reasons mentioned above.

Italy has an overwhelming responsibility for this disgraceful situation. The consequences of leaving EMBL for Italian science as well as for its public image will be very serious. We do not understand whether Italy is calling for an affirmative action employment policy by EMBL, or whether this is once more a confused, irrational government decision.

Italy seems to be blaming others for the results of its own corrupted politics. EMBL should not be blamed for the degradation of Italian science. We only hope that in the long run the changes in the Italian political system will eventually overcome these problems.

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Warm brown fat

SIR — The article by Lowell *et al.* (*Nature* 366, 740–742; 1993) deals with the genetic ablation of brown fat. For the ignorant, brown fat is what keeps us from freezing when the temperature dips below 0 °C. This weekend in Montreal, we suffered –31 °C. At least one of the authors of the Lowell *et al.* paper is a (misguided) Canadian. We take this opportunity to ask that she and her colleagues get their priorities straight. We need more brown fat not less.

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Nature, nurture and psychodarwinism

SIR — Part of the danger to which John Maddox draws attention in his remarks about genetic triumphalism¹ may be a result of lingering behaviourism — a school of thought distinguished by its emphasis on nurture (which it called 'conditioning') and its tendency to ignore evolution. As my colleague Helena Cronin has recently pointed out, Darwin's own approach to explaining human behaviour was 'psychological' rather than 'ethological' or 'sociological' because Darwin was "interested in our emotions rather than our actions"². A prime example was Darwin's view of "the coming of the sense of pleasure and pain as one of the most important steps in the development of mind"³. As David Barash has pointed out, what Darwin anticipates here is what Freud was later to call "the pleasure-unpleasure principle" — a psychological mechanism that demonstrably evolved to promote behaviour normally advantageous to the survival and reproductive success of the organism's genes and to discourage the converse⁴.

What Maddox calls the strong genetic principle is often suggested by a way of reporting scientific findings that links genes with measured behaviour without mention of the mind that mediates the two. But specifying nurture as another influence on behaviour does not remedy this neglect. On the contrary, it encourages a view of nurture as antithetical to nature without limiting the excess to which both sides can be driven in their dispute about which can best explain behaviour. By contrast, the pleasure-as-a-carrot-and-pain-as-a-stick model of genetic influence necessarily implies a decision-making agency that can be influenced by it. Our subjective experience of conflicts occasioned by the pleasure principle should remind us that we have choices to make and that those choices need not always serve it. If we add to this picture the influence of nurture on our conscience and nature on our appetites we arrive at a model of the mind that is an effective reply to both genetic and cultural triumphalism. As Darwin anticipated, the issue of how genes influence behaviour is ultimately neither a genetic nor sociological issue, but a psychological one.

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SIR — Maddox¹ draws attention to "the growing belief in a kind of genetic predestination". He says the lessons of 40 years of molecular biology may have "sunk in too well", creating in the public consciousness an oversimplified view of the genes as

biological programs determining every aspect of life.

Few scientists will disagree that nurture is of importance in developing a phenotype. It is, indeed, a subject thoroughly treated by undergraduate textbooks. Considering the limited possibilities for correlating a human phenotype with a specific genotype, "genetic predestination", meaning anything more than tenable statistical significance, is mere belief. However, this — in historical terms — dubious belief is not due solely to misleading popular science or "too zealous scientific advocacy of the importance of human genome studies".

It is a daily encountered fact that "life science" to a large extent has turned into "life technology". The advantage of this development is obvious. Vast economic resources in combination with scientific understanding of diseases has provided society with an efficient and widespread health-care system. But the chimaera of science and technology doesn't necessarily have the head of science and the forceful body of commercialism. Such curious signs, as pointed out in your article, of scientific research replacing prudence in interpreting results with "triumphalism", should be a warning. Genetic predestination has a tempting simplicity, appealing to those who are guided by another motive than that of understanding nature.

Despite the difficulties in interpreting genetic information, I fear that genetic predestination as a chosen model could have a renaissance in areas of commercial exploitation of genetic science. Attempts to re-establish the role of nurture by scientific discussion would do little to change this situation. A much broader debate on the interpretation of genetic information, concerning both scientific principles as such and their translation into a commercial context, is needed.

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SIR — It is sad to see Maddox¹ treat psychology with a cavalier disregard for empirical support that he would not dream of in relation to physics or molecular biology. He dismisses the discussions of the 1960s about the inheritance of IQ as though the arguments in favour of a strong genetic influence upon individual differences in intelligence had since been shown to be false. In fact, there is now general acceptance among serious workers in this field that those arguments were essentially correct⁵⁻⁸. To make matters worse, he then lumps together "a century of psychol-

ogy and psychoanalysis" as though these two disciplines had an equivalent empirical status. Would he do the same for astronomy and astrology?

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Helping Russia

SIR — Your readers should know of the benefits to Russian science of the operation of the International Science Foundation (ISF), known in the former Soviet Union as the Soros Foundation.

I am one of more than 25,000 scientists supported by the first wave of ISF activity in the form of emergency \$500 grants. I have seen how stimulating it was, both financially and psychologically. It is valuable to know that the international scientific community is concerned about the fate of Russian science.

With redoubled energy and optimism, many scientists were involved in the second step — preparation of proposals for large, scientific projects in competition for large grants over the next three years. And, again, what is very important is not only the financial support but also gaining it through grant competition, which is new and almost unprecedented for us. The competition is based on the international peer review system, and that will introduce international scientific criteria to our science.

Of no less importance is the support of our scientific libraries, which have become empty over the past few years, and support for international travelling by our scientists.

The ISF's activities are producing revolutionary changes in all aspects of our scientific life. I am most grateful to Mr George Soros for his most fruitful and wise programme.

Natalia Engelhardt

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

A fresh start for European science

I. Barry Holland

Instead of becoming embroiled in bureaucracy and the misplaced support of 'network' projects, the European Commission could provide an exciting, long-term science policy.

LET me declare my interest immediately. I strongly support the concept of a single European scientific community — who could not? — and I believe that the European Commission in Brussels can and must play a vital role in promoting this concept. Indeed, I have experienced and benefited from the Commission's efforts in my own research. But after more than 10 years of sponsoring projects, rather than individuals, the Commission's policy needs to be assessed.

This question is particularly crucial at a time when the European Union is floundering in its attempts to maintain the "European ideal". An exciting, long-term European science policy would provide the perfect opportunity for renewing the momentum towards greater integration. Rationalization of higher-education policy and the unrestricted freedom of movement of scientists throughout Europe are two areas that the Commission is in a position to promote with great effect.

In advocating such a policy, I have to say that Brussels, for all the undoubted dedication of the people working there, has been pursuing a science policy that it is ill-suited to carry out. I believe that the Commission should largely jettison its efforts to identify "winning projects", whether fundamental or applied, and should instead promote the concept of a sustained European science policy. The direct funding of projects should be left to national agencies, which are far better equipped to assess fundamental and applied science through hands-on experience and informed peer review.

The scientific arm of the Commission suffers the handicap of having no true roots in academic or industrial communities across Europe. As such it cannot be in tune with the pressing needs of scientific research or its commercial exploitation. Yet the Commission's scientific administrators develop frequent initiatives, no doubt with the highest of motives, in selected areas of basic or applied science, without the knowledge and experience of national agencies, which (quite rightly) are dominated by active scientists with close links with local academic and industrial sectors.

Collaborative bureaucracy

European Union initiatives have the laudable aim of promoting collaborations between member states, but how successful have these been? The Commission has

no way of taking the pulse of activity on the ground, or to anticipate the natural collaborations that are being held back for lack of funding or opportunity. Rather, proposals are tossed to the scientific community in the expectation that collaborations will automatically follow, which the Commission has divined as "good". The retort may be that scientists are only too happy to scramble for these delights — well, yes, we are. Scientists, particularly impoverished scientists, have become adept, chameleon-like, in adjusting to the requirements of the most specific projects imaginable. But what a degree of contortion this can necessitate — and what pain, effort and time can be taken to produce in some cases quite phoney collaborations! And this is the easy part. Unlike most national funding agencies with which I am familiar, access to European funding is overwhelmingly bureaucratic: contracts brim with legalistic and semi-commercial detail; cash takes an inordinate amount of time to arrive; and there is no understanding of the realities of recruiting staff and the complexities of managing modern scientific groups.

In my opinion, the unquestioned talents and vision of the Commission could be far more effectively used to promote the greater mobility of European scientists, which would lead to better collaborations than have so far been achieved. This should include greater mobility for graduate students, postdocs and for established scientists as individuals, not linked to cumbersome networks. Movement of graduate students between countries to complete a PhD is essentially zero. Movement of postdocs has improved, but demand greatly exceeds the available funding. Regular programmes to support short-term movement of established scientists are rare, and permanent migration is still greatly inhibited by nontransferable pension schemes, bureaucracy and national idiosyncracies.

I propose that the Commission devotes the bulk of its funding for the support of PhD students to study in member states other than their own; for postdoctoral fellowships; for short- and long-term visits for established scientists; and for greater support for multinational workshops and symposia. The procedures, while remaining accountable, must be greatly streamlined. Most important, such a programme must be sustained over a decade or more if it is to have real impact (why must

Brussels chop and change its initiatives so frequently?).

The Commission must back this policy with strong leadership to rationalize PhD training and career development throughout member states. The objective would be to produce more flexible and, above all, more rapid career progression to independent status for young scientists. The potential of talented individuals is being held back in many European countries by insufficient support or by suffocation from outdated academic hierarchies, which block mobility even within the national framework. Different member states, no doubt for different reasons, appear unable to deal with the key issues of graduate training or to appreciate the need for attractive, effective career structures. The Commission is in an ideal position to generate ideas and leadership.

Sustained effort

In terms of graduate training, the Commission could draw up guidelines for PhD training — putting its weight behind, for example, the growing consensus for a fully funded master's degree followed by a further 3 years' research training. These guidelines could remove many anomalies, such as costs incurred by non-nationals studying for a PhD (essentially nothing in France compared with several thousand pounds in the United Kingdom), guaranteed length of time for funding (3 years in the United Kingdom but only 2 years in France) and so on. Similarly, the Commission should look at career development: in some countries postdoctoral funding is essentially non-existent, resulting in enormous bottlenecks in people's careers.

The essence of my plea is that we desperately need a much greater concentration of effort on the training of young scientists and the removal of barriers, both physical and psychological, to easy movement for training and to subsequent career development. This must be a *sustained* programme with a minimum of bureaucracy. In my view the Commission, free of national political baggage, is in a far better position to implement such a policy than individual member states. National agencies, on the other hand, are best able to formulate policies for the stimulation of specific areas of science. □

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How to make taxol from scratch

Susan Band Horwitz

Devising a total synthesis of the anti-cancer drug taxol has engaged the attention of chemists for more than 20 years. But their ingenuity — and perseverance — has been rewarded.

THE total synthesis of taxol, announced by Nicolaou and colleagues on page 630 of this issue¹, is a scientific landmark and an intellectual achievement. Taxol is an anti-cancer drug of natural-product origin, and ever since its structure was published in 1971 (ref. 2) a complete synthesis has loomed as a major challenge for chemists. As John Mann describes in the accompanying box (overleaf), Nicolaou *et al.* have met with success by taking a new approach. It is not clear whether their accomplishment will make large quantities of the drug available to treat patients, or result in a decrease in its cost, but it will allow the preparation of new compounds that otherwise would be unavailable for testing and for studies on mechanism of action.

The history of taxol is highly instructive for those interested in drug development and its inherent difficulties. It is over 30 years since bark, twigs, needles and fruit of *Taxus brevifolia*, the Pacific yew tree, were collected in the north-west of the United States for a joint project sponsored by the US Department of Agriculture and the National Cancer Institute to search for new sources of anti-tumour drugs. Taxol is present in the yew's bark in minute quantities (the sacrifice of a 100-year old tree yields about 3 kg of bark containing some 300 mg of taxol, about a single dose), and its isolation in the 1960s and determination of its structure by Wall and Wani and collaborators² in itself was quite an achievement. The path from the forest through the laboratory to the patient has been successful because of the perseverance of a cadre of chemists, pharmacologists and oncologists — and because of the support of the National Cancer Institute.

Although taxol displayed good activity against human tumour xenografts and murine B16 melanoma, its cytotoxic properties were not very different from other drugs being tested during the 1970s (ref. 3). Rather, its attraction to pharmacologists was its unique structure, a complex diterpenoid containing the taxane ring system and a rare four-membered oxetane ring and ester side chain, that suggested the possibility of a new mechanism

for an anti-tumour drug.

This mechanism was subsequently identified. Taxol proved to be a potent inhibitor of eukaryotic cell replication, blocking cells in the late G2-M phase of the cell cycle. It is an unusual mitotic inhibitor

bule cytoskeleton⁴. The novel characteristic of taxol is its ability to polymerize tubulin *in vitro* in the absence of guanosine 5'-triphosphate (GTP), which is normally required for tubulin assembly⁵. It is the only drug known to bind at a specific site on the microtubule polymer⁶, and recent studies with a taxol photoaffinity analogue have identified the amino-terminal 31 amino acids of β -tubulin as the main site of photoincorporation⁷. The isolation and characterization of compounds with unique structures and mechanisms should lead to drugs with new spectra of clinical activity.

Although an understanding of taxol's mechanism of action stimulated a great deal of interest, its clinical development has been fraught with difficulties. The scarcity of the drug limited its availability for adequate clinical trials, and the hydrophobic nature of the compound has made it a continual challenge for formulation chemists, whose goal is to deliver compounds in aqueous solution. The supply problem⁸ has been overcome through the availability of precursors to taxol such as 10-deacetyl baccatin III, which can be isolated from the needles of various *Taxus* species and used for the preparation of semisynthetic taxol⁹. The needles regenerate, so they provide a continuous source of precursor (unlike the harvest of bark, which destroyed the trees and created an outcry among environmentalists, who were also concerned about the endangered spotted owl that lives in the ancient forests). Adequate supplies of taxol will also be derived from 2–3-year-old *Taxus* bushes that are being grown for this purpose.

An unexpected drawback that emerged

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Pharmacological fruit in the forest — the Pacific yew, *Taxus brevifolia*, from which taxol was originally isolated.

because, unlike vinca alkaloids and colchicine which inhibit microtubule assembly, it promotes the formation of discrete bundles of stable microtubules that result from the reorganization of the microtu-

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when taxol was first introduced into clinical trials was the high incidence of major hypersensitivity reactions that may have been related to the formulation of the drug in Cremophor EL. Although these hypersensitivity reactions have been overcome by premedication regimens and alterations in the administration of the drug, they delayed clinical trials by five years¹⁰.

Nonetheless, taxol has become an important new cancer chemotherapeutic agent, one of the first in a number of years. It has significant activity in drug-refractory ovarian cancer¹¹ and was approved for this disease by the US Food and Drug Administration in 1992. Other tumours for which taxol has shown activity are cancers of the breast¹² and lung^{13,14},

and melanoma¹⁵. Although it holds uncommon promise in the treatment of various cancers, it is not a panacea or a cure for cancer; rather, it is an active drug whose true clinical potential will be realized only with time. Its effectiveness in combination chemotherapy, with radiation, with growth factors or as adjuvant chemotherapy in breast cancer remains to be seen.

The synthesis carried out by Nicolaou *et al.* opens up possibilities for the development of new active anti-tumour drugs, for compounds with greater aqueous solubility, and for others with the ability to reverse taxol-resistance. It will lead to extensive structure-activity studies that will allow an examination of interactions

between different parts of the taxol molecule and the microtubule. Access to new derivatives and a second generation of taxoids will further our knowledge of microtubule structure and function, and should add impetus to the development of rationally designed anti-tumour drugs to interact with microtubules. Not least, the work of all concerned in the taxol story has focused attention on natural products as a source for drugs and has implicated the microtubule as a major target for cancer chemotherapeutic agents. □

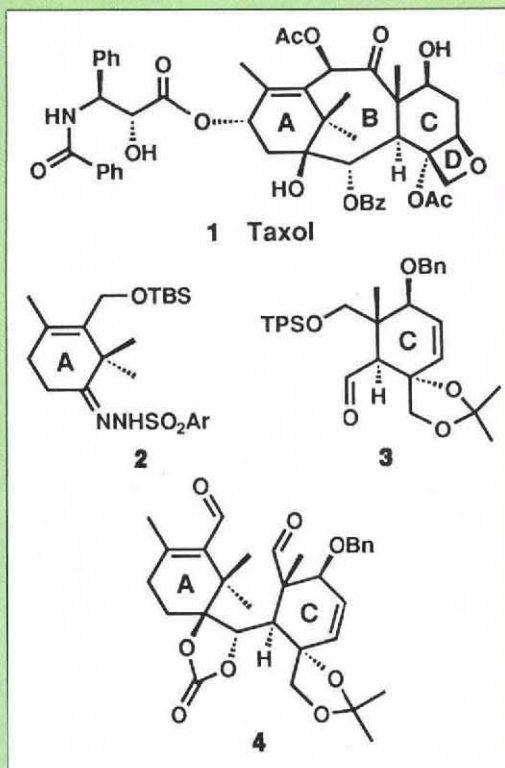
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Steps to a successful synthesis

In book VI of his *Gallic Wars* Caesar tells us that Catuvolcus, a chieftain of the Eburones, committed suicide by taking an extract of the yew tree. This was a common form of poisoning in ancient times, but the broad-spectrum cytotoxicity of extracts from the Pacific yew, *Taxus brevifolia*, was only discovered in 1964. Subsequent purification and structure elucidation of the major cytotoxic principle, the diterpene taxol (1), was achieved by Wani *et al.* in 1971, but clinical trials were delayed by the paucity of natural taxol available. Nonetheless, phase I trials in humans commenced in 1983 and the great promise of taxol was confirmed.

The growing interest in the anti-tumour potential of taxol, coupled with the synthetic challenge offered by a molecule of this structural complexity and rarity, attracted the attention of many of the world's leading synthetic chemists. Taxol rapidly became one of the most fashionable targets of the eighties. In a review written in early 1992, Swindell¹ provided a progress report on the work of more than 30 groups, but noted that there had been "only modest success in total synthesis". Two years on, we have the first total synthesis by Nicolaou and co-workers.

The molecule presents a formidable synthetic challenge with its eleven stereocentres and dense array of functionality. Nicolaou and colleagues' synthesis is both convergent — it employs the fully functionalized A-ring and C-ring fragments 2 and 3 (the former first prepared^{2,3} in 1992) — and flexible in that it should allow the construction of numerous analogues. The first carbon-carbon bond between rings A and C was created through reaction of the carban-



ion generated from sulphonylhydrazone (2) and aldehyde (3) (Shapiro reaction). Subsequent manipulation of functional groups provided the dialdehyde (4), and ring B was closed using a McMurry reaction mediated by titanium trichloride and activated zinc. The yield of this step was very low (23 per cent), but the rest of the synthesis, which included oxetane formation, oxygenation of ring A and introduction of the side-chain, proceeded without major problems.

There are 28 chemical steps based on the intermediates 2 and 3 (which also have to be prepared), so the synthesis can hardly be described as commercially viable. An alternative, shorter route by Wender's group, based on α -pinene, must be nearing completion⁴, and other groups are not far behind. Indeed, as these pages are finalized, a group led by Robert Holton at Florida State University has claimed, in a press release, that it has achieved a total synthesis. Details will appear in the *Journal of the American Chemical Society*.

Meanwhile, as Susan Horwitz describes, it has been shown that taxol and certain related structures, especially 10-deacetyl baccatin III, may be obtained from needles of the European yew, *Taxus baccata* (for a review, see ref. 5). And the demonstration that a fungal endophyte of the Pacific yew, *Taxomyces andreanae*, will produce taxol and other taxanes in culture⁶, offers some promise for the microbial production of these molecules.

It would be wrong, however, to underestimate the importance of the elegant synthesis developed by Nicolaou *et al.* It will allow access to structures not available from natural sources, some of which may be more potent than taxol. Structure-activity studies will help to clarify the mode of action of this fascinating molecule.

John Mann

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Replacing the solar flare myth

Nancy Crooker

EVERY eleven years on average, around the peak in the solar-terrestrial activity cycle, there are great magnetic storms which disrupt regional electrical power systems, interfere (sometimes fatally)

immense quantities (even by solar standards) of coronal material. Detectable only from space-borne coronagraphs — instruments that eclipse the Sun's bright disk — they appear as gigantic loop-like bubbles subtending up to a quarter of the Sun's circumference (see Fig. 1). The bubbles lift off into space, leaving behind only bright legs rooted to the Sun. As coronal mass ejections are space-age latecomers to the solar-terrestrial scene (they were discovered in the early 1970s), space physicists are only now appreciating their central relevance to the field.

So explained the session's lead speaker, Steve Kahler (Air Force Phillips Laboratory), who traced the history of studies that established a clear, although far from perfect, correlation between flares and aurorae, or more commonly between flares and the magnetic storms that accompany aurorae. The reason that the misconception about flares is so entrenched in solar-terrestrial physics, he argued, is that correlation was mistaken for cause, a textbook case of the famous logical

in opposite directions annihilate each other and release energy in the form of X-rays and energetic particles. Because the solar surface is constantly in motion, like a boiling liquid, and because its rotation rate is not uniform in latitude or with depth, its embedded magnetic fields are constantly shifting, deforming and twisting on a hierarchy of scale sizes. As a result, oppositely directed magnetic fields are frequently brought together, triggering reconnection and consequent flares. Coronal mass ejections involve a specific form of large-scale magnetic field deformation. As a mass ejection rises off the surface, it draws out the oppositely directed magnetic fields in the legs of its loop-like form, which can then reconnect and cause a flare. Thus flares can be side-effects of coronal mass ejections as well as a common phenomenon elsewhere on the Sun.

Don Reames (NASA Goddard Space Flight Center) corrected the misconception that flares produce large-scale expulsions of solar energetic particles. Although flares do impart energy to particles (possibly directly through reconnection or indirectly by generating instabilities and shock waves in the corona), the huge shock waves that fast coronal mass ejections push before them can energize particles on a much grander scale. Particles accelerated by these shocks can be distinguished from those associated with flares through measurements of composition and charge state. These show that flare-associated particles are limited to narrow cone angles, whereas ejection-association particles can span

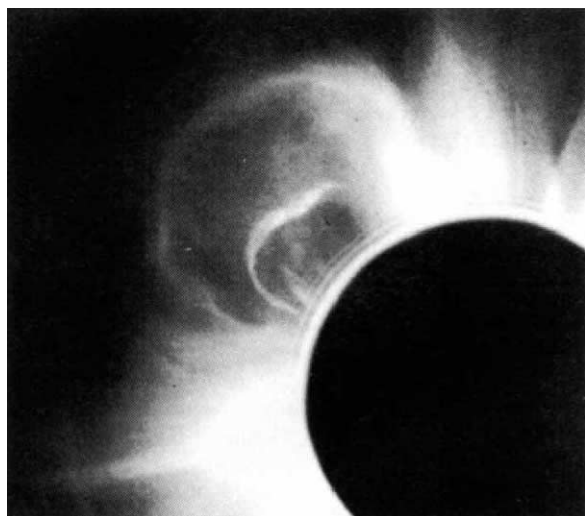


FIG. 1 A coronal mass ejection lifting off the Sun. The bright outer loop is the leading edge, and the brighter inner loop is the associated erupting prominence (A. Hundhausen, Solar Maximum Mission, NASA).

with commercial and government satellites, expose astronauts to radiation hazard, and spread aurorae from their polar home across the skies of temperate climes. Quick to respond, the news media consult experts at appropriate observatories, universities or government agencies and report, "Scientists say that last night's extraordinary display of the northern lights was caused by the explosion on the Sun of a violent solar flare", or, if they are science reporters, "by energetic particles from a violent solar flare".

Wrong, says Jack Gosling (Los Alamos National Laboratory), whose year-long campaign to spread a new gospel of solar-terrestrial lore culminated with the publication of his article, "The solar flare myth" (*J. geophys. Res.* **98**, 18937–18949; 1993), and the success of a session he organized for a meeting in December* entitled, innocently enough, "The solar-terrestrial connection". The article and session conveyed a revolutionary message: solar flares do not cause magnetic storms and attendant auroral displays, nor do they cause most of the associated, hazardous fluxes of solar energetic particles; coronal mass ejections do.

Coronal mass ejections are rapid reconfigurations of coronal structures that involve the ejection into the solar wind of

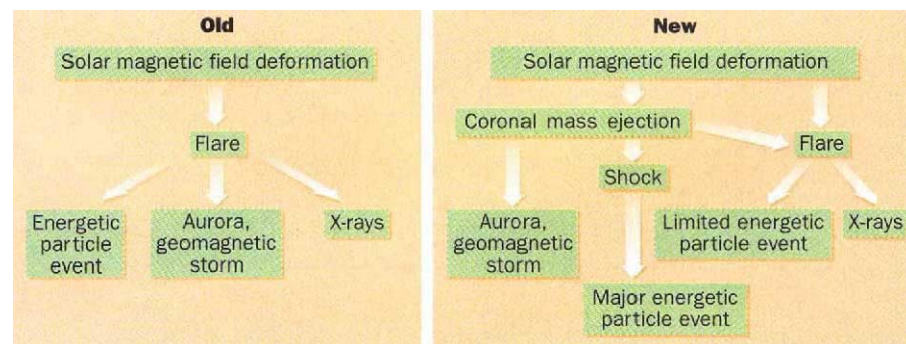


FIG. 2 The traditional and revised versions of the causal chain for solar-terrestrial events.

fallacy: *post hoc ergo propter hoc* (after A, therefore because of A). Usually establishing correlation is the first step towards establishing cause. But in the case of flares and aurorae, both are side-effects of a common cause, coronal mass ejections (see Fig. 2).

On the other hand, not all flares are caused by coronal mass ejections, as flares occur much more frequently. Art Hundhausen (NCAR High Altitude Observatory) explained that flares may be caused by 'magnetic reconnection', a process in which magnetic fields pointing

cones covering half of interplanetary space.

Jack Gosling adduced convincing evidence that coronal mass ejections cause all of the larger magnetic storms and accompanying aurorae. The properties of mass ejections responsible for storms are the strength and configuration of their magnetic fields, and their high speeds — not the energetic particles, contrary to most popular and some scientific accounts. The magnetic field in the quiet solar wind usually lies in the ecliptic plane. But the loop-like fields in mass ejections have

* Fall Meeting of the American Geophysical Union, San Francisco, California, 6–10 December 1993.

strong north-south components. As the Earth's magnetic field points northward, any oncoming southward fields reconnect with it; and the stronger the fields and the faster they approach, the faster the reconnection rate. Without reconnection, the Earth's field acts as a barrier to shield the near-Earth region, the magnetosphere, from the solar wind. But reconnection breaks the barrier and allows solar wind particles and energy to pour into the magnetosphere at a rate proportional to the reconnection rate. Through a series of magnetospheric processes, the energy gained drives the currents that make the magnetic storm and accelerates both the infiltrating solar wind particles and local particles to create the aurorae. So although it is true that aurorae are caused by energetic particles, they are locally energized and different from those associ-

ated with mass ejections and flares.

Thanks to Gosling, a new solar-terrestrial exhibit planned for the Smithsonian's Air and Space Museum in Washington DC will get the flare story right. Early exhibit plans that actually promulgated the 'solar flare myth' are what sparked his revisionist crusade. The exhibit now recognizes the central role of coronal mass ejections. Although solar flares are intrinsically interesting phenomena, and predictions of their occurrence are important for protecting space travellers from potentially lethal X-ray doses, we now understand that they do not cause aurorae, magnetic storms or major energetic particle events. □

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METEORITICS

Star of the small screen

David W. Hughes

EVERY year, many thousands of meteorites fall to Earth, but unfortunately for museums and meteorite collectors, the vast majority are never found. In contrast, the Peekskill meteorite¹ almost seems to have been trying to publicize its arrival, skimming low over a highly populated area of the United States before crashing to Earth through the rear of a car (shown in the photograph).

Meteorites are small asteroidal fragments that survive their transit through the Earth's atmosphere. This gaseous blanket retards them from a typical space velocity of 75,000 km h⁻¹ to a relatively benign 150 km h⁻¹, albeit with consider-

able surface mass loss and the production of prodigious heat, light, ionization and sound. Meteorite parents usually have masses in the 10 kg to 10⁶ kg range. Smaller bodies burn out in the atmosphere; larger ones punch through, producing craters in the Earth's surface and destroying themselves in the process.

Before the Peekskill meteorite fall, discussed by Brown and colleagues on page 624 of this issue¹, only three atmospheric trajectories had been photographed accurately enough for the Solar System orbit of the parent body to be calculated. Several photographs of the fireball trains were needed in each case.

From the observer's point of view, the Peekskill meteorite had many advantages. It hit the atmosphere on a near-grazing trajectory, giving the resulting fireball a visible path that was over 700 kilometres long. It was first seen over Kentucky and then moved north-northeast over West Virginia, Pennsylvania and New Jersey before finally landing in Peekskill, New York. The timing could hardly have been better. It fell shortly before 8:00 p.m. local time (23:48 UT) on 9 October 1992, when many people were outside watching high-school football games. Some had their camcorders with them. Startled by the vivid greeny-white ball of fire streaking across the sky, some momentarily forsook the football on the pitch and filmed the meteorite instead.

Brown *et al.* have analysed fourteen of the resulting videos. Frame by frame, they have been able to follow the fate of the incident meteorite as it ablated and fragmented. Never before has such a wealth of data been available. The incident body was seen to break up into about 70 pieces when it was 41.5 km above the ground. Four main fragments probably hit the ground, but the only one found so far is the 12-kg chondritic rock that hit Michelle Knapp's 1980 Chevrolet Malibu.

The orbit of the incident body was perfectly normal, having a perihelion distance of 0.886 astronomical units (1 AU is the distance from the Earth to the Sun) and an aphelion distance of about 2.1 AU, on the inner edge of the main asteroid belt. What was unusual, though, was its low angle of incidence. The most probable angle is 45° (refs 2, 3), but Peekskill came in at only 3.4° and nearly bounced off the atmosphere. The last well-recorded⁴ 'bouncing' meteorite was visible for over 100 seconds as it travelled the 1,500 km between Salt Lake City and Calgary on 10 August 1972, coming within 58 km of the ground.

Another fascinating characteristic of Peekskill, and one that owes its discovery to the videos, was that the luminosity of the incident parent flickered at a frequency of about 6 Hz before it fragmented. Maybe this was caused by the molten surface dripping off the parent body every 2 km along the flight path.

It is a pity that only one fragment has been found. Others are probably strewn over an 80 by 15 km field. As to the future, perhaps asteroid-watchers should stake out their Chevys as bait and see what happens . . . □

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When two worlds meet — the car that suffered a most unusual collision.

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VISION

No crossing at the crossing

R. W. Guillery

Two papers^{1,2}, to be published within a fortnight of one another, offer new insights into how the brain processes sensory events. Both report individuals in whom all fibres from the eye pass to the cerebral hemisphere without crossing the midline. The first paper, which will appear in the March issue of *The European Journal of Neuroscience*, is by Apkarian *et al.*, and is of interest because it deals with two children and gives an indication of how such abnormalities may be spotted. The second, by Williams *et al.* (page 637 of this issue), describes an inherited abnormality in sheep-dogs, offering greater scope for experimental, particularly developmental, studies.

These results bear on a topic with a rich history, for it has long been assumed that our brains need orderly maps of the outside world. Descartes argued that because our lenses reverse the image falling on the retinas, there must be a partial fibre crossing in the brain so that the two reversed images, one from each eye, can be brought into register in the pineal (Fig. 1; see ref. 3 for references). Newton³, also starting from the optics, proposed that there must be a partial crossing in the optic chiasm, and clearly described the optic chiasm as we now know it in primates. The underlying thought for both was that some surrogate 'person' in the brain must have a map if seeing was to be possible. Convincing experimental evidence about the chiasm was not obtained until the late nineteenth century. Although von Gudden⁴ (the psychiatrist who certified 'mad' King Ludwig of Bavaria, and subsequently drowned with the king in Lake Starnberg) identified the partial crossing in rabbits (as in Fig. 2c), it was some years before this pattern was accepted as characteristic of mammals. Birds such as the owl, in which all the fibres cross at the chiasm, remained a puzzle, until it was shown that a partial intracerebral crossing⁵ established a pathway rather like that proposed by Descartes. However, owls do not have the completely uncrossed optic nerves of Fig. 1, nor is the pineal still considered relevant to vision.

Thinking about sensory mechanisms continued under the implicit or explicit domination of a cerebral 'being' that needed maps. A striking example was Cajal's suggestion⁶ that the combination of reversing lens and the need for maps had led to each hemisphere being con-

nected to the opposite side of the body (see Fig. 2). His explanation has great appeal, and the accumulation of solid evidence for extraordinarily accurate motor and sensory (touch, sight, hearing) maps in the brain has seemed to confirm views that started out as assumptions

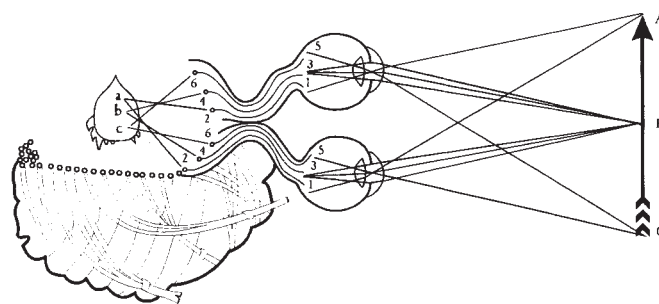


FIG. 1 Schema proposed by Descartes for bringing two orderly mapped representations of the visual field into register with each other in the pineal gland. Because the lens reverses the image, a partial crossing must occur somewhere to allow a superposition of the images of the two arrows. Descartes placed the partial crossing in the brain, Newton at the optic chiasm. (Figures 1 and 2 redrawn by Terry Richards from the originals.)

about perception.

Figure 2 shows that if, as in the children and dogs now described, the entire optic nerve goes to one hemisphere without crossing, there must be a partial map reversal. If, for the mammalian system shown in Fig. 2c, the abnormal pathway maps with normal topography but on the wrong side, then the arrow head would

map to regions normally occupied by the tip of the tail, producing a reversal.

The G in Cajal's drawing (Fig. 2c) represents the main relay to the cortex, the lateral geniculate nucleus. This is now known to consist of two sets of stacked layers, not the single continuous layer shown. The stacked layers are shown in Fig. 4 of the paper by Williams *et al.*² (page 639), where the abnormal reversal is evident (4b).

How does the brain deal with such reversals? The observation that children and dogs show a nystagmus (an involuntary oscillatory movement of the eyes) demonstrates that subcortical centres controlling eye movements do not compensate for the reversal. This is important because in albinos, who also have an abnormality of the crossing pattern^{1,2,7} and a nystagmus, it has never been easy to blame the abnormal crossing for the nystagmus; albinos also have other abnormalities of central vision. The children in the study by Apkarian *et al.*¹ may demonstrate a clear link between the nystag-

mus and the reported low visual acuity on the one hand, and the pathway abnormality on the other, thus also providing a useful tool for identifying the abnormality in other species.

The cerebral cortex must be smarter than the subcortical centres. The children report normal visual fields, and our knowledge of the albino mutation sug-

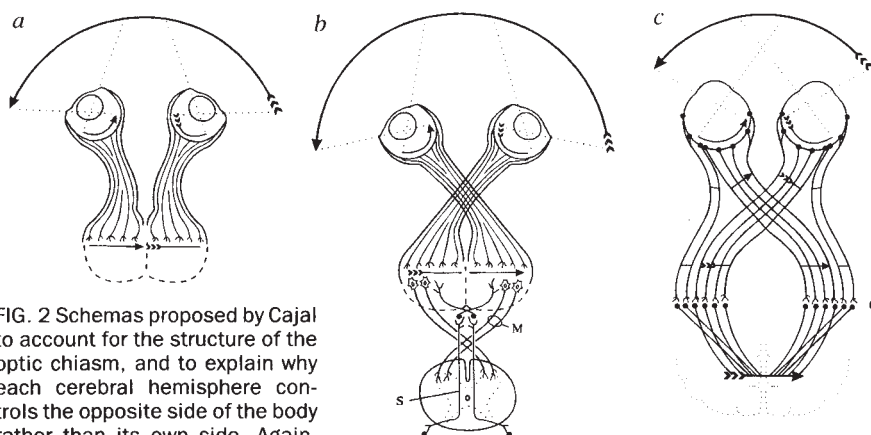


FIG. 2 Schemas proposed by Cajal to account for the structure of the optic chiasm, and to explain why each cerebral hemisphere controls the opposite side of the body rather than its own side. Again, the problem is set by the reversing lens, which in *a* produces a broken arrow in the brain. *b*, How in vertebrates the problem is resolved by a crossing in the optic chiasm. Now the left hemisphere sees the right part of the world, and the motor (M) and sensory (S) pathways have to be crossed to accommodate the visual crossing. *c*, What happens when the eyes face forward and both eyes share a part of the visual field. Now some of the fibres cross, others stay on their own side. This is the situation in most mammals. The G in Cajal's figure is the lateral geniculate nucleus, the main relay on the way to the cortex. Here Cajal showed a broken arrow which he puts right by a further crossing on the path to the cortex. We now know that this relay is not a single continuous layer as in *c*, but two sets of stacked layers, arranged so that the small piece of each arrow lies directly opposite the visually corresponding part of the long piece (see Fig. 4a, page 639). The geniculocortical crossing is not needed in a normal animal, but has been demonstrated in animals having an abnormal chiasm^{8,9}.

gests that the cortex can deal with the mirror reversal, either by keeping the two maps, one a reversal of the other (one black, one white in Fig. 4c of the paper in this issue) in two separate cortical domains, or else by rewiring the connections going to the cortex so that the numerical sequence running from 8 to 1 in Fig. 4c is re-created as a continuous sequence in the cortex^{8,9}. The C-shaped fusion of geniculate layers in the dogs points to rewiring, but the critical evidence is not available. The experiment in dogs is simple and is, no doubt, being done.

Developmental mechanisms producing the modified geniculocortical pathways are likely to prove of particular interest, as establishing two independent cortical domains almost certainly depends upon visual experience. By contrast, rewiring the geniculocortical pathway is a prenatal event, independent of vision^{9,10}.

Perhaps a most telling point in support of the assumptions about maps made over 300 years ago is that a map like that in Williams and colleagues' Fig. 4c, which is part black and part white, containing an

internal reversal, cannot be established in the cortex unless one part is very small, or the reversal is corrected^{7,10}. The internal inconsistency seems to fool the cortex, as Descartes, Newton and Cajal thought it should. However, against their expectations, the brain has ways of dealing with total reversals. □

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MATHEMATICS

Parsimonious polyhedra

Jeremy Gray

AN age-old problem about the most economical packing of space has just received a new solution. The question is: what space-filling arrangement of cells of equal volume has minimum surface area? In this month's issue of *Philosophical Magazine Letters*, D. Weaire and R. Phelan announce that they have been able to improve on a solution of Lord Kelvin's that has stood for 107 years. Their new

proposal, illustrated here, does better by a margin of 0.3 per cent, for which they modestly claim "a remarkably large margin of superiority in this context".

The single surface that encloses the greatest volume for a given area is, of course, the sphere. But spheres do not pack together without gaps. Over the centuries, different teams have entered the competition to fill space without gaps;

of these the bee is surely the best known. Praise for the skill with which it makes its combs with a minimum amount of wax goes back at least as far as the Greeks. Pappus wrote "The bees know just this fact which is useful to them, that the hexagon will hold more honey for the same expenditure of material than the square and the triangle". Indeed, the solution in the plane is the regular hexagon, displayed in cross-sections of a honeycomb.

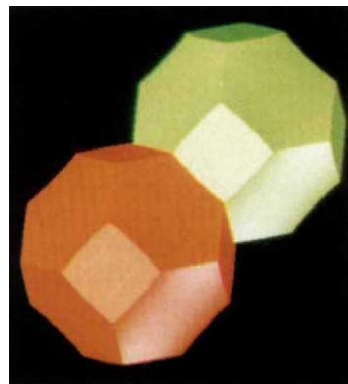
But the world is three-dimensional, and an even better solution is to go back to the spheres and start to pack them together. When this is done layer by layer, it becomes clear that spheres in the second layer will not sit exactly on top of spheres in the layer below, but over the gaps. In this off-centre position, each sphere will have 12 neighbours: three in the layer below, six in its own layer, and three in the layer above. Something of the kind is seen where the two layers of cells on a honeycomb meet back-to-back. What the bees do is let surface tension cap off their hexagonal cells while the wax is soft and place the right-facing caps in one layer in the dimples between the left-facing caps in the other layer (see Scientific Correspondence, D. Weaire and R. Phelan *Nature* **367**, 123; 1994).

There is no simple way to fill the gaps mathematically, so ingenuity is called for. Stephen Hales, the distinguished early biologist and contemporary of Newton's, had the bright idea of squashing peas in a barrel. He found that the result was an irregular looking solid, with individual peas being deformed to shapes with some 13 or 14 sides.

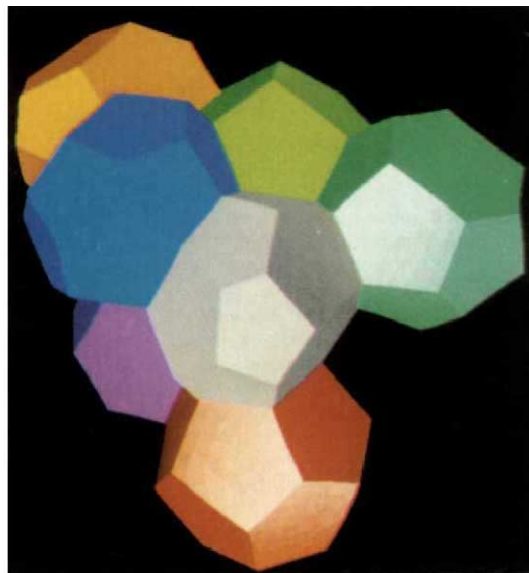
Hales's work does not yield a single space-filling polyhedron. So one turns to nature's next most attractive candidate, crystal lattices, to look for shapes that can be carried round to fill space in a lattice pattern. Apart from the cube, no other regular solid will fill space. But among the semi-regular solids (known to Archimedes) the rhombic dodecahedron does fill space. Indeed, it may be thought of as the solid obtained by systematically filling in the gaps in the layered arrangement of spheres described above.

What Lord Kelvin did was to take the semi-regular solid that is obtained from an octahedron by slicing off its six vertices at a certain distance from each vertex. This 14-sided figure does fill space, and the crystallographer Fedorov had exhibited natural examples of it. Kelvin showed that by curving its sides and faces so that it was a little more like a sphere, one can obtain an even better solution than the bee's. And there the matter rested for over a century.

Weaire and Phelan's new improvement is again inspired by inanimate nature, in this case dry foam. The space-filling cubic



How to fill space most efficiently. Left, the solution proposed by Lord Kelvin — identical 14-sided polyhedra, with subtle curvature of the faces and edges to minimize the surface area. Right, one unit cell of the space-filling structure proposed by Weaire and Phelan; the polyhedra have identical volumes, but six are 12-sided and two are 14-sided. Only seven of the eight polyhedra are visible here. Again, the faces and edges are gently curved (images generated using the GEOMVIEW package).



clathrate structure $\text{Na}_8\text{Si}_{46}$ is taken as the starting point, and used to generate a space-filling array of 14-sided cells of unequal sizes. The sizes were then equalized by using the 'Surface Evolver' computer package of K. Brakke published some 12 years ago. It equalizes the volumes while minimizing the surface area of each cell. A particular feature of this package, which runs on a work station rather than a supercomputer, is that it enables the original flat cell walls to be gradually curved. The area of each curved wall is estimated by taking it to be composed of steadily larger numbers of small flat pieces (the planes can just be seen on the polyhedra in the figure). In this way,

Weaire and Phelan were able to design a new cell shape and show that it improved upon Kelvin's long-standing record holder.

The problem is still not wrapped up. The authors themselves have other candidates still to investigate and, as they point out, candidates are still coming forward on an *ad hoc* basis. Filling space economically is one of those mathematically formulated problems for which there is still no mathematical theory capable of providing a systematic approach. □

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PROTEIN TRANSPORT

On the beaten pathway

Bernhard Dobberstein

TRANSPORT of proteins across bacterial plasma membranes is evolutionarily related to transport of proteins across the endoplasmic reticulum (ER) membrane in eukaryotic cells. In both systems, proteins destined for secretion are usually synthesized as precursors with a hydrophobic core signal sequence. At first, however, it looked like the respective targeting and translocation machinery would turn out to be very different — although the so-called signal recognition particle (SRP) and its receptor mediate targeting of nascent secretory proteins to the ER membrane, two structurally unrelated proteins, SecB and SecA, guide secretory proteins to the plasma membrane in bacteria¹. But as more detail emerges, so that view is having to change. For example, papers by Miller *et al.*² and Hartmann *et al.*³ (pages 657 and 654 of this issue) indicate that there is not only a striking similarity between the pro- and eukaryotic signal-recognition and protein-targeting apparatus, but also between the translocation sites. The implication, of course, is that the mechanisms involved must have much in common.

In eukaryotes, a ribosome synthesizing a secretory protein is targeted to the ER membrane by the sequential interactions of the signal sequence on a nascent polypeptide with SRP and the SRP receptor (SR) in the membrane (see figure). The SRP is a ribonucleoprotein composed of the 7S RNA and six polypeptides with relative molecular masses of 9,000–72,000 (see table). The signal sequence of the nascent

secretory protein is recognized by the 54K protein subunit of SRP (SRP54). Contact between SRP and the signal sequence reduces the rate of polypeptide-chain

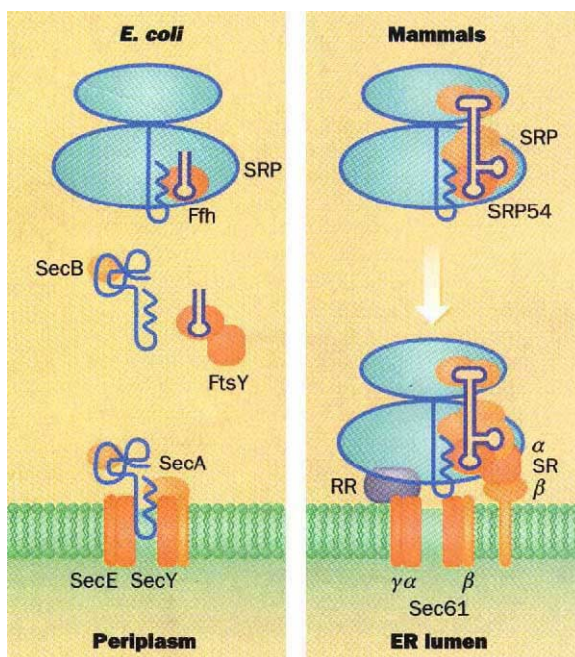
SRP54, the SR α and probably also the SR β subunits. *In vitro*, targeting and translocation requires the presence of GTP⁶. GTPases in a wide variety of molecules function as molecular switches which confer unidirectionality and specificity on biochemical processes. In the case of SRP-mediated protein translocation, cycles of GTP binding and hydrolysis appear to regulate the interactions of nascent secretory proteins with the targeting and translocation machinery. How exactly the GTPases work in the context of the ribosome nascent chain complex is not yet known. But from analysis of complexes formed between purified SRP or SRP subparticles and SR, some principles have emerged. For instance, GTP binding to SRP54 increases upon contact with SR (ref. 7), and if this holds true for the complete system then SR α acts as a 'guanine nucleotide loading' protein which promotes GTP binding to SRP54. SR itself probably needs to bind GTP in order to stimulate GTP binding to SRP54 (ref. 8).

Somewhat surprisingly, Miller and colleagues previously found that the presence of a signal sequence prevents

SR from stimulating GTP binding and hydrolysis. This probably indicates that one or more additional components are needed for promoting GTP binding when a signal sequence interacts with SRP54. As GTP is required for signal-sequence release, it is likely that the additional factor ensures that the signal sequence is released from SRP only when appropriate components of the translocation site are available^{5,7}.

Molecules structurally similar to SRP 7S RNA, SRP54 and SR α occur in *Escherichia coli*, namely 4.5S RNA, Ffh (also known as P48) and FtsY respectively⁹ (see table). As 4.5S RNA and Ffh form a ribonucleoprotein complex that specifically recognizes nascent polypeptides containing a signal sequence, the complex has been termed an SRP¹⁰. Miller *et al.*² now show that this SRP and FtsY communicate in like manner to their mammalian counterparts. In the presence of non-hydrolysable guanine nucleotides, *E. coli* SRP and an FtsY derivative form a stable complex, and GTP hydrolysis is significantly stimulated when they are combined. Miller *et al.*² find that, as with the mammalian

proteins, FtsY is not able to stimulate GTP hydrolysis in the presence of a functional signal sequence. This suggests that bacteria have a signal-recognition and protein-targeting system that functionally resembles the SRP and SR system of eukaryotic cells. Furthermore, it seems



Factors involved in signal-sequence binding, targeting and membrane translocation in *E. coli* and mammalian cells. The signal sequence is shown as a zig-zag. Proteins that are similar in all organisms are in red, and those for which no counterpart is known yet in brown. RNA (4.5S RNA and 7S RNA) is shown as a thick purple line separated by a space. RR, ribosome receptor.

elongation. Upon contact with the SR, the signal sequence is released from SRP, elongation proceeds and the nascent chain inserts into the ER membrane through a proteinaceous pore^{4,5}.

The process of targeting to the ER membrane is regulated by GTPases in

that in *E. coli*, as in mammalian cells, factors besides SRP and FtsY are required for the release of the signal sequence from SRP and successful targeting. These could be an as yet unknown SRP homologue, a chaperone or components of the translocation site.

Protein components of the translocation site were first identified by genetic screens in *E. coli*¹¹, then in the yeast *Saccharomyces cerevisiae*¹², and most re-

branes. Similarly, mammalian SR and the Sec61 complex reconstituted into lipid vesicles promote protein translocation^{14,15}. It therefore seems that the core components of the translocation site have been characterized and consist of the pore protein, Sec61/Y, and associated proteins. Two small proteins associate with mammalian Sec61 and *E. coli* SecY, but until now only unrelated proteins have been found to associate with yeast

SEC61p and it has been suggested that two of them (SEC62p and SEC63p) are involved in signal recognition and targeting¹². So we can expect further growth in the number of proteins that are found to be in, or associated with, the translocation site. For instance, little is known about the release of the nascent chains from the translocation site. In *E. coli*, such a function could conceivably be performed by SecD and SecF.

No eukaryotic homologues for the *E. coli* SecB and SecA have yet been found. These two proteins interact during the targeting of some secretory proteins to the plasma membrane. It is perfectly possible that they constitute an alternative targeting pathway to the SRP/FtsY system, a

notion which finds support in the fact that deletion of Ffh does not affect the secretion of SecB-dependent proteins¹⁶. In yeast there are also strong indications for alternative targeting pathway(s) involving the heat-shock protein HSP70 and possibly SEC62/63p¹². So it seems that as well as the common pathway represented by SRP/SR and Sec61/Y, species- and substrate-specific secretion pathways have also evolved. □

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DAEDALUS

Deep insight

THE ocean floor is less well known than the surface of the Moon. At present, a few isolated patches can occasionally be glimpsed by robot submersibles. Daedalus now suggests a new approach. He is devising an undersea vehicle to crawl along the existing submarine cables.

This neat trick has many advantages. Each cable has a known diameter and surface texture, so a matching drive mechanism can be designed to grip and traverse it. The cable could easily transmit signals to the crawler, and pick up its results, by induction (spy submarines used to read cable traffic in this way). Even coaxial and fibre-optic cables could communicate with the crawler inductively via the power leads for their repeater-amplifiers. A signal sent along the cable to the crawler would take time to reach it, and time to return. This delay would give its exact position on the ocean floor.

For some of its length, a cable may be buried in the mud of the ocean floor, either by the impact of laying or subsequently by slow deposition of detritus from above. The crawler's drive would have to be powerful enough to push this overburden aside. It would also need a certain flexibility to negotiate splices and amplifier pods in the cable, and marine organisms such as shellfish which might be growing on it (though these should be rare in the depths). The crawler would project some sort of buoyant mast upwards to survey the ocean bottom from above the opaque muddy clouds raised by its passage.

The power supply for the crawler poses problems. Its inductive coupling to the cable could only provide it with milliwatts. It might burn carbonaceous fuel in the ocean's dissolved oxygen, as a fish does. But Daedalus prefers a metallic fuel which slowly dissolves in the surrounding water and generates electricity directly, in battery fashion. With certain chemical precautions, lithium seems the metal of choice. Being lighter than water, it adds no weight to the crawler. A fairly modest supply should power it across the widest ocean.

Cheap and effective, cable crawlers will transform oceanography. Along every cable, a succession of crawlers will map the ocean floor and log its changing fauna. Not only will they transmit images and data back to shore; on command they will gather specimens for later study. But careful traffic control will be needed. Two crawlers which met on the same cable could never get past each other. At least one would have to let go, develop buoyancy, and rise to the surface to await rescue. David Jones

Factors involved in protein secretion in mammalian cells, yeast and <i>E. coli</i>			
	Mammals	Yeast	<i>E. coli</i>
Cytosol (signal recognition, chaperoning)			
SRP-RNA	7S RNA	SCR1	4.5S RNA
SRP proteins	9, 14, 19 SRP54 68, 72	SEC65 SRP54 ?	Ffh (P48) SecB
Membrane (docking)			
SRP receptor (SR)	SR α (DP) SR β (DP) ?	SR α ? ?	FtsY ? SecA
Membrane (translocation)			
Translocation machinery	Sec61- α Sec61- β Sec61- γ	SEC61p ? SSS1p	SecY ? SecE band 1 SecD SecF

cently by a biochemical approach in mammalian cells⁵. Strikingly, the central part of the translocation complex in all three systems seems to consist of related proteins. Using crosslinking approaches, Sec61- α of mammalian cells, SEC61p of yeast and SecY of *E. coli* have been shown to line the postulated translocation pore⁵ (see figure). Structurally, these proteins are quite similar.

In all systems, other proteins associate with Sec61/SecY. These are Sec61- β and Sec61- γ in mammalian cells, SecE and band 1 in *E. coli*, and SEC62p, SEC63p and SEC66p in yeast. Mammalian Sec61- β and Sec61- γ have now been isolated and sequenced by Hartmann *et al.*³. Both proteins are predicted to span the membrane once. Of particular interest is that mammalian Sec61- γ bears significant homology to SSS1p of *S. cerevisiae* and can functionally replace it; SSS1p protein was discovered last year as a suppressor of *sec61* temperature-sensitive mutants¹³. Sec61- γ also has a small, but probably significant, resemblance to the SecE protein of bacteria.

Reconstitution studies have shown that, in *E. coli*, SecA and SecY/E are the only membrane proteins required for translocation of a pre-protein across mem-

Allomyces in the Devonian

SIR — We have found fossil fungi morphologically similar to *Allomyces*, the modern experimental fungus, in thin sections of early Devonian (400 million-year-old) megaplants. The fungus exhibits an isomorphic alternation of generations in which sporothalli that produce zoosporangia and resting sporangia alternate with gametothalli that form chains of gametangia. This life history is identical to that of the *Allomyces* subgenus *Euallomyces* and underscores the diversity in thallus morphology and reproduction in early terrestrial zoosporic fungi.

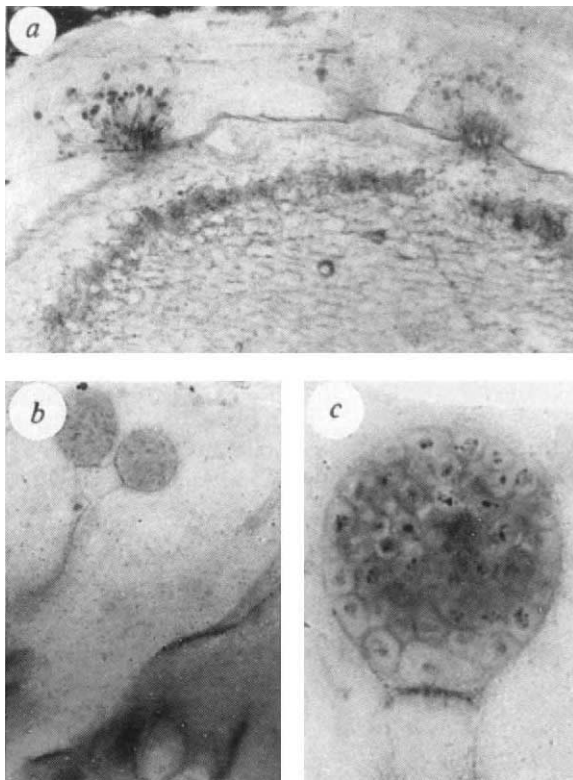
The fungus was saprophytic on *Aglaophyton major*, an early Devonian land plant, and occurs in small tufts on the surface of stems (*a* in the figure). It consists of nonseptate hyphae each 9–18 μm in diameter, which develop into two types of morphologically identical thalli (*b*). On one type of thallus (sporothallus) are terminal zoosporangia (*b*) and thick-walled resting sporangia. Zoospores are ellipsoidal, approximately 5 μm in diameter, and have a dark inclusion (*c*) which may be equivalent to the prominent nuclear cap of living blastocladiacean zoospores¹. The other thallus type (gametothallus) produces pairs of barrel-shaped gametangia in which the terminal one is always slightly larger.

The fossil fungus is most similar to living members of the Blastocladiaceae, a group regarded as more derived than other Chytridiomycetes. The presence of isomorphic thalli, each forming different reproductive structures (zoosporangia and resistant sporangia on sporothalli; gametangia of two distinct types on gametothalli), demonstrates that this fossil fungus possessed an isomorphic alternation of generations, a life-history pattern that has been documented in only a few living fungi.

Despite the investigation of various characters including zoospore flagellation², ultrastructure³, thallus morphology⁴, nutritional mode⁵ and ribosomal RNA sequences⁶, there is no consensus regarding the evolution of the Chytridiomycetes. The most comprehensive phylogenetic analysis⁷ places chytridiaceous fungi in three distinct groups: Spizellomycesales–Chytridiales–Monoblepharidales; Neo-

callimastales; and Blastocladiaceae. Similarly, there is no agreement regarding the origin of the Blastocladiaceae from other chytrid groups³, because these same chert deposits also contain other chytrids that morphologically resemble members of the Spizellomycesales^{8,9}. Nevertheless, because the Rhynie chert specimens represent the oldest fossils attributable to the Blastocladiaceae, they provide the only method available to calibrate molecular-clock datasets used to infer the phylogeny of fungal groups. Finally, the presence of this fungus by early Devonian time not only underscores the antiquity of the order but, perhaps more important, demonstrates that thallus morphology and reproductive systems in some terrestrial

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Allomyces-like fossil fungus. *a*, Partial transverse section of *Aglaophyton major* stem showing fungal tufts. $\times 40$. *b*, Sporothallus with terminal zoosporangia (left) and gametothallus (right) with pairs of gametangia. $\times 400$. *c*, Detail of zoosporangium containing cleaved zoospores with dark inclusions. $\times 1,200$.

fungi were established very early, and apparently have changed little since then.

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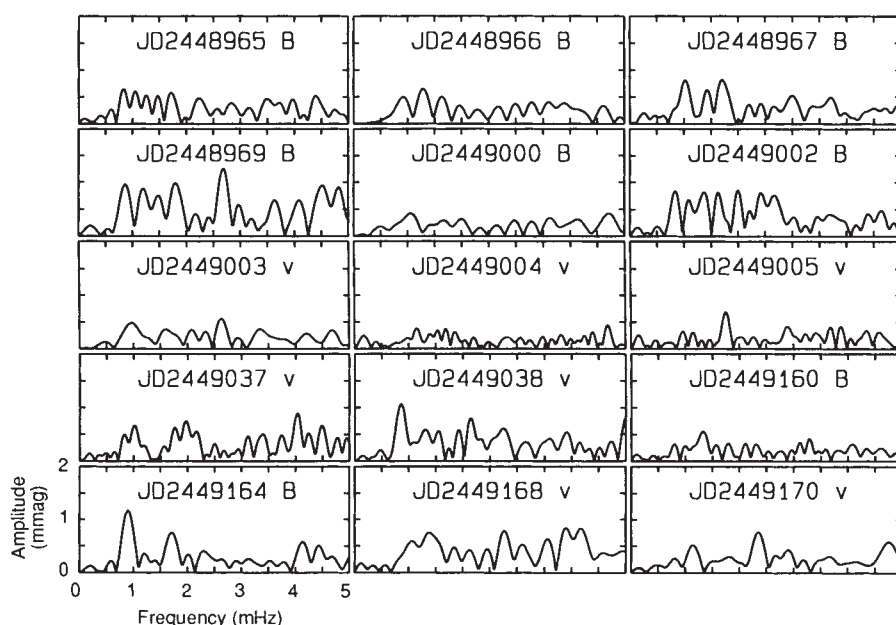
Fast pulsations in Wolf–Rayet stars

SIR — The interesting report¹ of the discovery of rapid pulsations in a Wolf–Rayet star suggests that asteroseismology can be used to study the interior structure and dynamics of these stars shrouded by hot, optically dense stellar winds in the same way as it is used to study the Sun² and other distant stars³. Here we report a significant null result in our attempt to confirm the existence of rapid oscillations with a period of 627 s in the Wolf–Rayet star WR40 (HD96548).

We acquired high-speed photometric observations of WR40 on 15 nights spanning 205 days from 9 December 1992 to 1 July 1993 using the 0.5-, 0.75- and 1.0-m telescopes of the South African Astronomical Observatory at Sutherland (see table). The programme star was monitored continuously over a time span of 1–2 h without any comparison star measurements. The reliability of this technique is proven for variability in the period regime of interest here⁴. The Fourier transforms of our 15 light curves are shown in the figure. In general, the noise level in our transforms is somewhat lower than that reported in ref. 1, but there is no evidence of coherent oscillations at 1.6 mHz. Although the figure shows only the frequency range $0 \leq \nu \leq 5$ mHz, we have examined the transforms up to the Nyquist frequency of our data (12.5 mHz) and verified that there are no statistically significant peaks in the first Nyquist interval.

A caveat we must mention is that the Johnson *B* and Strömgren *v* filters we used pass different amounts of emission line flux compared to the Geneva *B* filter used in ref. 1. This is an important consideration because the variations (or lack thereof) are attributed to the continuum, which arises in deeper regions than the emission lines. The Strömgren *v* filter approximates a Geneva *B* filter fairly well.

It is worth noting that the 627-s oscillations were detected using a charge-coupled device (CCD) photometer with no on-frame comparison stars. All earlier searches for rapid oscillations in this star



Fourier transforms of the 15 light curves listed in the table computed using Deeming's⁸ algorithm. The ordinates are scaled in amplitude (half the peak-to-peak variation), not power. Each panel is 0.002 mag high. The Julian date and filter used are listed in each panel. Some low-frequency ($\nu \leq 0.6$ mHz) sky transparency variations have been removed.

(and other Wolf-Rayet stars) by Blecha *et al.*¹ and others^{5,6} using conventional photoelectric aperture photometers produced null results. Blecha (personal communication) has informed us that recent attempts by his collaborators to confirm the 627-s oscillations in WR40 using their CCD photometer have also yielded null results. Thus, the possibility of a spurious detection cannot be excluded.

Barring instrumental artefacts and statistical 'false alarms'⁷ as the origin of the 627-s oscillations in WR40, our null result indicates that either the oscillations are modulated in amplitude or the circumstellar material surrounding WR40 is rarely sufficiently transparent to permit the oscillations to be observed. Although a pure radial mode is not excluded, the

observations presented here suggest that if WR40 is really pulsating and if these pulsations are multiperiodic or modulated in some way, it will be extremely difficult to obtain a thorough knowledge of its pulsation spectrum for asteroseismological studies. For the moment, however, the challenge facing observers is to confirm the reality of the oscillations reported by Blecha *et al.* Oscillations of the amplitude reported are readily detectable with a 0.5-m telescope at a good photometric site. Given the astrophysical significance of a confirmation of the oscillations and the modest requirement of telescope aperture, observers are encouraged to cultivate an interest in this star. A 1-h run a few times per observing season is recommended.

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8967.52270	1.51	134	B	2.0
8969.45066	1.23	109	B	3.3
9000.55618	1.01	90	B	1.0
9002.53937	1.57	139	B	1.9
9003.54648	1.27	113	V	1.3
9004.52269	2.25	194	V	1.2
9005.51903	1.96	173	V	1.5
9037.56211	1.85	154	V	2.4
9038.48507	1.64	145	V	2.1
9160.19928	1.96	173	B	1.3
9164.22631	1.36	117	B	1.6
9168.27892	1.33	119	V	2.5
9170.19782	1.15	97	V	1.4
Σ	22.75	1981		

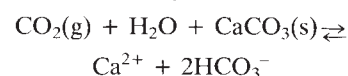
HJD, heliocentric Julian date; *t*, duration of observations; *N*₄₀, number of 40-s observations; Filt., filter used (Johnson *B* or Strömgren *V*); σ , standard deviation of one observation with respect to the mean.

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More on tea scum

SIR — Although we agree with Jones¹ that the brown stain on tea (see ref. 2) is formed by the adsorption of tannin from tea on the surface of the film of calcium carbonate that settles on teapots, where does the solid film of CaCO₃ come from?

CaCO₃ precipitates when hard water is boiled, due to the expulsion of dissolved CO₂. The solubility of CaCO₃ from limestones in water is caused by its reaction with hydrolysed carbon dioxide, forming the soluble calcium bicarbonate (see equation below). When hard water containing this calcium bicarbonate is heated, the expulsion of CO₂ shifts the equilibrium of the equation to the left, leading to the precipitation of CaCO₃.



The solid CaCO₃ is the familiar fur formed inside kettles and heating pipes in hard-water regions. We believe this is the primary cause of formation of the scum that floats on the surface of the teas prepared with hard waters.

Very simple experiments can reinforce this conclusion. If only hard water (with no tea) is boiled for a few minutes, a white scum is also formed on the surface (the process is easier to observe if a dark container is used). Alternatively, if tea is infused in distilled water in a clean beaker, no scum is formed, as already pointed out by Spiro and Jaganyi². We have observed this results in the United Kingdom using either Milton Keynes or Southampton tap water supplies. In addition, we have never observed tea scum when the same kind of teas are prepared in Brazil, where the water from tap supplies is typically soft. Consequently, we do not agree with Lewin³ that the major components of the scum on tea are high-melting point lipids.

It is also worth noting that CaCO₃ is a well-known adsorbent⁴, particularly in the amorphous state, which can be expected to be the predominant form during the boiling of hard water. We can thus expect a strong adsorption of the tannin from tea by CaCO₃, giving rise to the coloured sticky scum.

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Closing Pandora's box

Laurence Martin

A Nuclear-Weapon-Free World: Desirable? Feasible? Edited by Joseph Rotblat, Jack Steinberger and Bhalchandra Udgaonkar. Westview Press: 1993. Pp. 228. £33.50, \$52.50.

THE end of the Cold War and the subsequent eruption of more acute if lesser conflicts have pushed nuclear questions off the top of the security agenda. Admittedly, for experts and specialists, the problem of proliferation has perhaps become more acute, partly because of fears about the future of former Soviet forces and in part simply because the mere passage of time is bringing more possible candidate nuclear powers up to the necessary level of technical competence. For the public, however, the previously unthinkable progress in agreement between Russia and the United States to reduce their nuclear arsenals, together with the officially approved line that Russia is no longer an antagonist, has made the questions of nuclear posture, which in the guise of SALT and START used to dominate the international agenda, now seem like yesterday's business.

It is the great merit of this Pugwash Monograph to ask where all this supposed progress is leading us. Are the nuclear powers merely 'downsizing' forces which are to be the permanent basis of a balance of power or are they on the way to abolishing them, thereby attaining what has long been their ostensible but hitherto unrealistic goal of a denuclearized world? Although achieving such a goal of total nuclear disarmament would, as the authors all admit, be a task for decades, if only because of the physical problems involved, the question of the ultimate goal is given urgency because denuclearization is already pledged in the Non-Proliferation Treaty (NPT), up for review in 1995.

The subtitle of this book, "Desirable? Feasible?", is well chosen. Given the long-declared preferences of many of the no fewer than 26 cosmopolitan contributors, the reader is not surprised to be told that the answer is 'yes' on both counts. On the whole, the feasibility study is more convincing, given its assumptions, than that of desirability. The discussion of the demanding and intrusive verification systems that would be required to give the necessary degree of reassurance is very well done, particularly in a long chapter by Theodore Taylor, and many of the diffi-

culties and risks are lucidly spelt out there and elsewhere, as in a consideration of the "break-out problem" by Marvin Miller and Jack Ruina. The task is rendered more challenging but not, apparently, impossible by the assumption of continued use of nuclear power for generating

the lay reader can spend a few intriguing minutes extrapolating in various directions from Frank Blackaby's assertion that "physical scientists, concerned as they are with natural laws that know no national boundaries, should not find any difficulty with the idea of loyalty to the international ideal of the total abolition of nuclear weapons" (p.12). Further discussion leads to the conclusion that probably no military secrecy at all could be permitted and that inspection should be possible anywhere, at once, without notice. Yet, as is also pointed out, some degree of confidentiality, for example about intelligence capabilities themselves, may serve on the side of the 'angels'.

Further difficulties are revealed when the authors turn, not very thoroughly, to the question of enforcing agreements. On this, as on some other questions, there is no unanimity in the book; for that reason it is very necessary to read the whole volume and not be content with Blackaby's helpful but necessarily incomplete introductory summary. One chapter admits that the agreement could not be enforced against a large power: "large states should not, we argue, be the object of all-out military measures to deal with a violation of a ban on nuclear weapons. Small states might be. There can be no disputing the charge of unfairness" (p. 141). Realistic perhaps, but also fraught with political difficulty. It is not hard to imagine such an international regime being resisted as one in which smart, high-technology conventional weaponry of the type used in the Gulf War perpetuates a Northern, probably American-led, hegemony.

How serious such questions would be and, indeed, how difficult and intrusive the verification system need be, depends very much on the answer to the other question of desirability. Obviously the two questions interact: if a non-nuclear world is highly be-

neficial, the wish to violate it will be less, and the more the powers can be assured it is a non-nuclear world, the more they may believe in its benefits. The basic assumption of the authors is that the evolution of nuclear strategy even before the end of the Cold War was making it clear that all nuclear weapons could do was to provide assurance against nuclear weapons. That explains the beginning of a rundown in arsenals even before the Soviet collapse and thus the absence of any practical objections to abolition provided that it is universal.

However, if one believes, as some do, that the existence of nuclear weapons

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Missiles away.

electricity, including a plutonium cycle. This analysis implies that the problem of nuclear weapons should not be regarded as an insuperable objection to such civilian uses.

There are, however, some very debatable issues. Believing that technical means of detection would not be adequate in themselves, the authors are keen advocates of "societal" inspection: that is, the encouragement of "whistleblowers". The political consequences of this requirement across a range of politics and cultures are a subject for some speculation. Scientists are seen as the best qualified for such tasks; this is no doubt correct, although

exerts a constraining influence over all confrontations between nuclear powers, then the old fear that denuclearization makes conventional war more likely has to be faced. Could Western Europe face the potential relapse of Russia into overbearing ways so calmly today if there were not still a nuclear stand-off? Some of the authors of this book seem to sense this, though it is nowhere explicitly accepted. Instead, doubts about the stability of a denuclearized world are answered by assertions that the world is in any case about to become rather peaceful or, less optimistically, that such political evolution must be accepted as a prerequisite for denuclearization. Thus "the world in which a Nuclear-Weapon-Free World was possible would be a world that was already partially demilitarized", or, still more extremely, "unless we reach and have confidence in the continuation of an unprecedented state of tranquillity among nations, with very strict controls on *all* weaponry, breakout fears might well lead to much greater instability than we now have and the very purpose of having a Nuclear-Weapon-Free World would be negated. That is, if hostile relationships develop in a Nuclear-Weapon-Free World, a nuclear arms race might develop that would be both more intensive and more likely to lead to nuclear use than if nuclear stockpiles were maintained at minimum levels." (p. 83).

This is quite a comedown; an extreme version of the long-accepted view that rearmament races may be less stable than primary arms races. The way the authors

as a group seem to duck this problem is to assert that the way to a nuclear-weapon-free world leads down the same road as that to minimum deterrence and is already mapped out for a long way by the sweeping agreements already arrived at by Russia and the United States. Perhaps that is largely true and we can use the time to pursue the issues that this book so usefully raises. But at various points on the journey it may prove dangerous to be travelling under a false prospectus. One of the earliest moments at which discomfort may occur is at the NPT review, for doubts about the sincerity of the nuclear powers are already long established. Moreover, which goal one sets certainly affects one's attitude to the three or four undeclared nuclear powers. The difference may also show up particularly early and embarrassingly for such lesser nuclear powers as Britain, whose nuclear forces are already minimal and which may therefore be especially vulnerable to loss of political support if they are increasingly seen as conceptually obsolescent.

Although sad but lesser issues seize the headlines, we must not ignore the lurking nuclear problems until some crisis pushes them under the nose of a dangerously ill-prepared world. The authors of this book may have underestimated the flaws in their analysis and the objections to their prescription, but they have certainly reopened some important questions. □

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It's a gas

David Archer

Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Edited by James G. Ferry. Chapman & Hall: 1993. Pp. 536. £60, \$75.

METHANOGENS release about 500 million tonnes of methane into the atmosphere annually. The methane, from sources as diverse as belching cows, termites, wetlands, paddy fields and landfills, is produced by methanogens degrading organic matter in anaerobic environments. Concern about the impact of methane on our environment is one reason to be interested in James Ferry's book. The book's strength and purpose, however, lie in an altogether different direction because it brings together authoritative and comprehensive descriptions of the biology of methanogenesis, the first book of its kind.

The methanogens are the best-studied group of Archaeobacteria. Although they are united by their ability to derive energy from methane production, they are in many other ways a diverse group of microorganisms with different species capable of growth, for example, at temperatures ranging from less than 10 °C in some sediments to the scalding temperatures (over 100 °C) of hydrothermal vents. Working with the methanogens has never been easy, but developments have reached the point where culture on a large scale is now routine. I enjoyed reading Ralph Wolfe's historical overview of methanogenesis, which put into context the chapters that followed. My only quibble is the sloppy editing: on one page alone, 26 references were cited and in 6 cases there was a discrepancy with the citation at the end of the chapter.

David Boone, William Whitman and Pierre Rouvière present a phylogenetically consistent taxonomy for the methanogens in a bold chapter that may prove to be as well cited as the seminal review by W. E. Balch *et al.* (*Microbiol. Rev.* **40**, 260–296; 1979). Following on, there are chapters on features of methanogens revealed by microscopy, physiological ecology of methanogens, eight consecutive chapters on various aspects of biochemistry and a final chapter on genetics of methanogens. Such is the division between chapters that, for example, the appearance of gas vesicles is described in one chapter but their biological significance, if any, is discussed in another. More often, however, some repetition between chapters was presumably necessary to allow chapters to be read in isolation.

The capacity of methanogens to derive energy from the production of methane is inherently interesting because it requires a



THE "flurry" — "The whale boat darts through the water in [the whale's] wake, the waves rising up like walls of glass on either side". Whale boats were made from Eastern redcedar wood because it is brittle; upon impact it punctures rather than splits, allowing the crew to plug the hole. From *New England Natives: A Celebration of People and Trees* by Sheila Connor. Harvard University Press, £31.95, \$47.95.

unique biochemistry and mechanisms for conserving energy. The bulk of the book focuses on these issues. Mechanisms for production of methane from known substrates, the biosynthesis and role of coenzymes unique to the methanogens and anabolic reactions are all described. Following advances made in understanding the biochemistry of methanogenesis came detailed studies of energy conservation carried out principally in the laboratory of Gerhard Gottschalk. The chapter by him and colleagues summarizes present knowledge and unique features of energy conservation by methanogens but also presents a view on mechanisms still requiring confirmation.

The final chapter by John Reeve deals with the structure and organization of genes in methanogens. This aspect of methanogen genetics has made rapid

progress in comparison to the development of efficient methods of gene transfer. The latter area is not included in Reeve's chapter although, as acknowledged in the editor's preface, gene transfer holds the key to rapid advances in many of the areas discussed throughout the book. Wolfe saw fit to end his 'historical overview' with the report of P. Schönheit that methanogens can now be grown under non-methanogenic conditions. This intriguing finding, taken together with the potential application of molecular genetic techniques, will mean that future editions of this book, excellent though it is, will need to accommodate much new information. □

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ground' explanations that attempt to incorporate aspects of both. He does, however, devote an entire chapter to 'mitochondrial Eve'. Such separate treatment not only has the advantage of decoupling this contentious issue from the palaeontological underpinnings of the 'single origin' view, but it also allows space for the most lucid general review of this complex topic I've seen.

The archaeological record shows clearly that the achievement of modern anatomy and of modern behaviour were not the same thing. Having dealt with anatomy, Lewin thus devotes the second half of his short book to the archaeological record and its interpretation. As he notes, recognizing 'modern' behaviour (whatever that is) from the archaeological traces of long-vanished populations is a far from simple task. Archaeologists differ, indeed, as vociferously as palaeoanthropologists do. One might expect that, delving into the complexities of cave art and the origins of consciousness and language, Lewin would display the same sympathy with those who believe in a late and rapid acquisition of the unique human capacity as he does in earlier chapters with proponents of a single origin for anatomically modern humans. Instead, he remains more detached, and this detachment gives the second half of his book a more muted tone than the first — although it's nonetheless a very useful rendition of the complexities of the ongoing debate.

Episode of human evolution

Ian Tattersall

The Origin of Modern Humans. By Roger Lewin. *Scientific American Library/W. H. Freeman: 1993. Pp. 204. \$32.95, £18.95.*

YET another book on modern human origins, currently the hottest topic in human evolution? Inevitably, yes. But with a difference. This one is by a single author, in contrast to the recent crop of edited volumes in which the usual pundits are trotted out at chapter length, in a remarkable variety of permutations and combinations. For Roger Lewin, a shrewd and seasoned journalistic observer of the palaeoanthropological scene, has taken it upon himself to present to the general reader a lavishly illustrated summary of the present state of play in this contentious field.

Actually, Lewin's book resembles the edited works to the extent that he has interviewed most of the aforementioned pundits, and quotes from many of them at some length. But despite his admirable attempts to present all sides of the argument, and his observer's reluctance to project his own sympathies too vigorously, Lewin makes no pretence of hiding these sympathies — which lie with those who believe that *Homo sapiens* originated in a single place, rather than in a worldwide process. Letting them out of the bag helps to give Lewin's book a focus that the edited volumes lack, and makes for an altogether more satisfying read.

To provide a context for his account of the continuing debate, Lewin starts right at the beginning. He succinctly reviews the history of discovery and analysis of the entire human fossil record, and the intrusion into its study of molecular systematics,

functional anatomy, archaeology and so forth. He is particularly good at evoking the way in which theoretical expectations have, throughout, affected interpretation of the evidence. Appropriately, he dwells at greatest length on the Neanderthals who, apart from being perhaps our closest extinct relatives, are the archaic human species that was most un-

arguably replaced by modern forms.

The background thus set, Lewin proceeds to contrast the two major competing viewpoints on modern human origins, and to summarize the evidence for each. Implicitly recognizing that the 'single origin' and 'multiregional continuity' models of modern human emergence are products of totally different and irreconcilable views of how the evolutionary process operates, he wastes little time on the 'middle-

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Where do we go from here? From the film *The Man Called Flintstone*.

Lewin has produced a readable and well organized account of the most important and most recent, yet most mysterious, episode in human evolution. It's an excellent introduction for the general reader, and many professionals will want it for the wealth of ideas aired in its pages. □

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Ghost in the machine

Harry Collins

The Fourth Discontinuity: The Co-evolution of Humans and Machines. By Bruce Mazlish. Yale University Press: 1993. Pp. 272. £22.50, \$35.00.

IF I had to bet on what would be a hot academic topic in the next 5 to 10 years, it would be the 'eternal triangle' — the relationship between humans, animals and machines. Neural nets are the latest cycle of renewed hope for those who believe that the borderline between humans and machines is fading, while some apes do seem to have developed levels of

being] . . . impossible that there should be enough different devices in a machine to make it behave in all the occurrences of life as our reason makes us behave" (Descartes quoted on p. 23).

In the first part of the book, Mazlish takes us through automata and the Industrial Revolution, with a quick look at how the ideas worked themselves into myths, such as the Golem, and into fictional characters such as Frankenstein, the associates of the Wizard of Oz, and Isaac Asimov's robots. The contributions of Linnaeus, Darwin, Freud and Pavlov to the mechanization of the idea of humankind and the establishment of the continuity between humans and animals are covered in the first two chapters of part two of the book. Chapter Seven discusses Babbage, Thomas Huxley and provides a particularly interesting exposition of Samuel Butler's thoughts on machines.

The Ronald Grant Archive

Part three of the book brings us up to date with biogenetics and 'artificial intelligence'. Mazlish's title, *The Fourth Discontinuity*, is taken from Jerome Bruner's idea that cognitive history comprises the establishment of three great continuities: the Greeks established that all matter, heavenly or earthly, was continuous and governed by the same laws; Darwin helped us to understand that humans are biologically continuous with animals and all other life forms; and Freud established that the infant and the adult, the mentally ill and the mentally healthy, primitive civilization and evolved civilization are all based on the same fundamental human drives. These, according to Bruner and Mazlish, were three great 'smashings of

the ego'; in each case humankind learned that its place in the Universe is less special than it believed. The fourth ego-smashing is to be the establishment of our continuity with machines.

Grand sweeps of argument like these are best delivered with hindsight. In the case of the fourth discontinuity the work is still to be done. The final chapters, which are supposed to deliver the promise, are disappointing. They are hedged about with many a "could be", "potential" and "if Marvin Minsky is right". That game is too easy to play; it tells us too little about machines and too much about coffee-table chat at the Massachusetts Institute of Technology. We already know that if Marvin Minsky is right the corners of the eternal triangle will soon be changing places and my computer will keep me as a

pet, but we also know that Minsky thought language understanding was a topic for a student summer project.

There are lots of ways for those coming from the humanities and social sciences to approach the eternal triangle. A more ambitious historical approach would analyse the way in which the establishment and destruction of the various boundaries in specific locations at specific times served people's interests. In the case of machines we might look closely at, say, the church's special interest in the soul, or capital's interest in diminishing the perceived uniqueness of human skills. A contemporary study in this vein would ask not 'what is the truth of the matter?', but 'who is using Hubert Dreyfus?', 'who is using Marvin Minsky?' and 'how does this affect the reception of their ideas?'. Another avenue is to try to bring to the debate something special that has been overlooked by the psychologists, engineers and mathematicians; something that only those from the humanities and social sciences understand about people and their knowledge. This requires that one engage to some degree with the technicalities of the field and try to contribute to technical progress. A handful of social scientists, of whom Lucy Suchman is the most obvious example, have had some success here; Mazlish seems ignorant of their work.

I find the last chapters of this book worrying because of the lack of ambition they express for the role of the social sciences. Mazlish is content to 'reveal' the vision of some of his more technologically accomplished colleagues without engaging in or with the arguments. □

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Identity crisis — man or machine?

language understanding that would be impressive in a one-way 'Turing test'.

Not all books that discuss hot topics are themselves hot, of course. Mazlish's book is most useful as a work of reference: a brief encyclopaedia of what various historical figures have already written on the subject. Thus, we soon discover how hard it is to say anything on the topic that has not already been covered, at least in outline, by Descartes: ". . . if there were a machine which had such a resemblance to our bodies, and imitated our actions as far as possible, there would always be two absolutely certain methods of recognizing that it was still not truly a man. The first is that it could never . . . modify its phrases to reply to the sense of whatever was said in its presence, even as the most stupid men can do. The second . . . [relies on it

New in paperback

The Cosmic Code: Quantum Physics as the Language of Nature by Heinz R. Pagels. Penguin, £7.99. See *Nature* 296, 503 (1982).

Metals in the Service of Man by Arthur Street and William Alexander (10th edn). Penguin, £8.99.

The Dance Language and Orientation of Bees by Karl von Frisch, translated by Leigh E. Chadwick. First published in 1967, this groundbreaking book is now issued with a foreword by Thomas D. Seeley. Harvard University Press, £23.95, \$35.95 (pbk).

Gates: How Microsoft's Mogul Reinvented an Industry and Made Himself the Richest Man in America by Stephen Manes and Paul Andrews (updated edn). Touchstone/Simon and Schuster, \$14.

Molecular and cellular mechanisms of general anaesthesia

N. P. Franks & W. R. Lieb

General anaesthetics are much more selective than is usually appreciated and may act by binding to only a small number of targets in the central nervous system. At surgical concentrations their principal effects are on ligand-gated (rather than voltage-gated) ion channels, with potentiation of postsynaptic inhibitory channel activity best fitting the pharmacological profile observed in general anaesthesia. Although the role of second messengers remains uncertain, it is now clear that anaesthetics act directly on proteins rather than on lipids.

ALTHOUGH there is little agreement about how and where general anaesthetics act, there has been an explosion of studies over the past decade on the effects of anaesthetics on putative targets in the central nervous system (CNS). Partly for historical reasons, but also because of the central role membranes play in nerve conduction, attention has focused largely on nerve membranes, and in particular on neuronal ion channels and the systems that regulate them. General anaesthetics probably exert their effects on synaptic transmission rather than on axonal conduction, but which synapses are the most sensitive? Do anaesthetics principally inhibit excitatory synapses or potentiate inhibitory synapses (or both), and are the crucial targets presynaptic or postsynaptic? Moreover, do anaesthetics bind directly to proteins or influence activity by indirectly perturbing membrane lipids, and to what extent do intracellular factors modulate sensitivity? Here we try to answer these questions, concentrating on work that over the past ten years has seemed to us to be not only the most definitive but also the most pertinent in pointing the way forward.

Voltage-gated ion channels

Na⁺ and K⁺ channels. The interactions of many simple organic compounds with the Na⁺ and K⁺ channels involved in action potential generation have been described in great detail for the squid giant axon¹. For the Na⁺ channel, most anaesthetics cause depolarizing shifts in the steady-state activation curve and hyperpolarizing shifts in the steady-state inactivation curve. At concentrations relevant to general anaesthesia (Box 1), however, these shifts are very small, and, as the delayed rectifier K⁺ channel is similarly insensitive^{1,2}, axonal conduction is virtually unaffected. Another voltage-gated channel, K_A, which is thought to play a role in regulating axonal firing, has also been proposed as a possible anaesthetic target³. This suggestion was made because mutants of *Drosophila* with dysfunctional K_A channels are less sensitive to isoflurane³. This change in anaesthetic potency, however, most probably arises from a generalized increase in neuronal excitability, because direct measurements on the K_A channel show little effect at relevant anaesthetic doses of halothane⁴ or the *n*-alcohols⁵. Although it is conceivable that small perturbations of voltage-gated channels may alter patterns of neuronal firing^{6,7}, it appears unlikely from the available evidence that voltage-gated Na⁺ or K⁺ channels play a substantial role in the production of the anaesthetic state.

Ca²⁺ channels. Voltage-gated Ca²⁺ channels (currently labelled T, L, N and P) share sequence homologies with voltage-gated Na⁺ and K⁺ channels and belong to the same superfamily. T- and L-type channels are present in a wide variety of both excitable and non-excitable cells, but N- and P-type channels have been found mainly in neurons. To date, most anaesthetic studies have focused on T- and L-type channels, rather than on the N- and P-types. But the latter two types of Ca²⁺ channel, particularly the P-type, appear to be most directly involved in

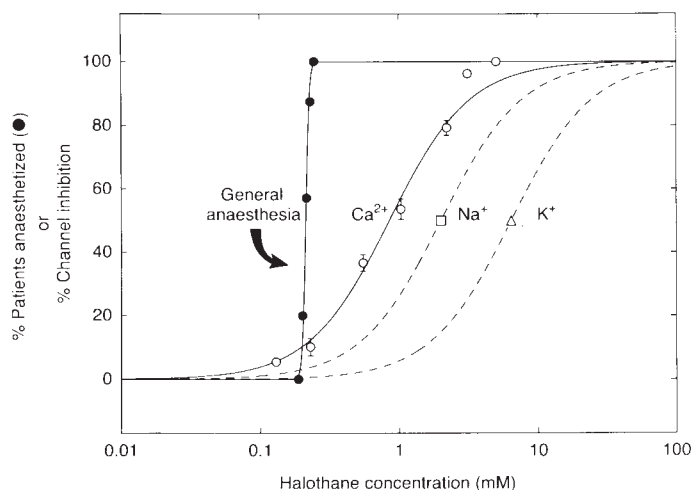


FIG. 1 Most voltage-gated ion channels are relatively insensitive to general anaesthetics. General anaesthesia in humans (●), as measured by the lack of a purposeful response to a surgical incision¹²⁸, occurs at concentrations of halothane 4 to 30 times lower than the EC₅₀ concentrations needed to half-inhibit peak currents through L-type Ca²⁺ channels (○) from clonal pituitary cells¹²⁹. Na⁺ channels (□) from the squid giant axon² or delayed rectifier K⁺ channels (△) from the squid giant axon². Note the extremely steep dose-response curve for general anaesthesia. Aqueous concentrations for general anaesthesia were calculated from partial pressures¹²⁸ using known water/gas partition coefficients. Dose-response curves for the ion channels were constructed using the Hill equation with EC₅₀ concentrations for the Ca²⁺, Na⁺ and K⁺ channels of 0.85 mM¹²⁹, 2.0 mM² and 6.4 mM², respectively, and a Hill coefficient¹²⁹ of 1.5. The dashed lines for the Na⁺ and K⁺ channels reflect the fact that only the EC₅₀ values are known, and for simplicity we have assumed the Hill coefficients to be the same as that for the Ca²⁺ channel.

the presynaptic release of neurotransmitters from central nerve terminals⁸. The inhibitory effects of volatile anaesthetics on Ca²⁺ channels have recently been reviewed⁹; perhaps surprisingly, most studies show that these channels are either very or moderately insensitive to these agents. An apparent exception is a recent report¹⁰ of exceptionally high sensitivity to halothane of a small low-voltage-activated Ca²⁺ current in rat sensory neurons. Barbiturates also inhibit Ca²⁺ channels^{11,13}, but usually with half-maximal inhibitory concentrations (IC₅₀) many-fold in excess of the free aqueous concentrations that cause general anaesthesia (Box 1). For example, the inhibitory effects of six barbiturates on N-like currents were studied¹³ in *Xenopus* oocytes injected with RNA from human brain; substantial effects were found only at very high barbiturate levels (for example, for pentobarbital, IC₅₀ ≈ 1 mM and 0.4 mM for peak and sustained currents, respectively). As nerve terminal Ca²⁺

channels open only briefly when an action potential invades a terminal, inhibition of the peak current is probably the more physiologically relevant measure of channel activity.

Overall, then, voltage-gated Ca^{2+} channels appear to be generally insensitive to clinically used general anaesthetics. It seems unlikely to us that the small inhibitions sometimes observed at surgical concentrations (Fig. 1) are sufficient to produce general anaesthesia (though the steep dependence of synaptic transmis-

sion on Ca^{2+} entry leaves this possibility open¹⁴). Moreover, some synapses appear to be unaffected by relevant levels of anaesthetics (see ref. 6, for example). Another approach used to assess the relevance of Ca^{2+} channels in anaesthesia relies on measurements of anaesthetic potencies in animals. For example, in both mice¹⁵ and dogs¹⁶, administration of Ca^{2+} -channel-blocking drugs reduces the anaesthetic requirement. Although it might be concluded that Ca^{2+} channels must therefore play a role in anaesthesia, this view must be set against the general insensitivity of these channels to anaesthetics. This illustrates the weakness of the approach, because changes in anaesthetic potencies in animals can so easily be caused by parallel effects on pathways quite distinct from those targeted by the anaesthetics themselves.

Ligand-gated ion channels

Glutamate receptors. L-Glutamate is probably the major excitatory neurotransmitter in the vertebrate CNS. Its ionotropic receptor channels (iGluRs), which mediate fast excitatory transmission, can be classified according to their selective agonists: NMDA (*N*-methyl-D-aspartate), KA (kainate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionate; formerly QUIS (quisqualate)). There have been surprisingly few reliable modern studies with clinical general anaesthetics on iGluRs, but a good case can be made that the dissociative agent ketamine exerts its anaesthetic effects largely by inhibiting the NMDA receptor^{17,18}. Ketamine is not only relatively selective for NMDA receptors¹⁸, but its stereoselectivity in whole animals (Box 2) is mirrored *in vitro* at the NMDA receptor^{17,19}. In contrast, all iGluRs appear to be relatively insensitive to volatile agents. For example, glutamate-activated currents in dissociated neurons from the rat nucleus tractus solitarius are unaffected by 300 μM halothane or enflurane, and the most anaesthetic-sensitive iGluR responses (to QUIS) are less than 40% inhibited by 1 mM halothane²⁰. Although halothane minimum alveolar concentration can be substantially reduced by selective AMPA²¹ and NMDA²² antagonists, the insensitivity of their iGluR targets argues against a major role in halothane anaesthesia. Conflicting reports exist for barbiturate inhibition of iGluRs, perhaps as a result of use-dependent block of non-NMDA receptors, as recently found²³ in cultured rat cortical neurons. In these neurons, pentobarbital was very effective at inhibiting KA currents ($\text{IC}_{50} = 50 \mu\text{M}$ pentobarbital), but only after prior activation by KA. QUIS responses were more complex, with peak currents resistant but steady-state currents reduced to half by 100 μM pentobarbital. NMDA currents were very insensitive to pentobarbital²³. Finally, it has been reported that ethanol selectively and, in common with other small aliphatic alcohols, potentially inhibits NMDA receptors²⁴, but this selectivity has recently been questioned²⁵ with data showing that KA/AMPA receptors can be just as sensitive to ethanol, especially at low agonist concentrations.

Nicotinic acetylcholine receptors. The nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction is the most thoroughly studied of all neurotransmitter receptors. Less is known about neuronal nAChRs, although they do share considerable sequence homologies and also function as pentameric oligomers. Work with general anaesthetics has inevitably concentrated on the more accessible muscle-type receptors. One of the few generalizations that can be made (although one probably irrelevant to anaesthesia) is that most simple anaesthetics, at high enough concentrations, are able to stabilize a desensitized form of the receptor²⁶. At more relevant doses, however, other effects are observed that vary markedly between agents. Among the volatile anaesthetics, the fluorinated ethers isoflurane and enflurane are particularly effective at inhibiting the receptor^{27,28}, with the primary effect at the single-channel level being to reduce the mean open time²⁹. Inhibition of a molluscan nAChR by isoflurane is stereoselective²⁸; the *S*(+) isomer is more effective than the *R*(-) isomer, as found for general anaesthesia in mice

BOX 1 Which anaesthetic concentrations are pharmacologically relevant?

GENERAL anaesthetics are administered in both the gaseous and aqueous phases. A problem encountered with inhalational agents is that EC_{50} s expressed as partial pressures (usually as % atm.) are extremely temperature-dependent, with the gas-phase potencies almost invariably increasing as the temperature is lowered. In fact, an anaesthetic partial pressure determined for a mammal at body temperature can be as much as three times too high for an experimental preparation at 20 °C (ref. 9). But as there is usually a parallel (although slightly smaller) temperature-dependence of water/gas partitioning, values of free aqueous concentrations (C_{water}) for a given anaesthetic are relatively independent of temperature. For example⁹, if 250 μM halothane is used (C_{water} calculated at 37 °C; see table), this will be only about 25% too high for an experiment at 20 °C. Recommended free aqueous concentrations for five inhalational agents are given in the table; these EC_{50} values are for 37 °C but are reasonable approximations for room-temperature experiments.

Estimating appropriate concentrations for intravenous agents poses different problems. These arise from their relatively rapid redistribution and metabolism, as well as from their high degree of binding to blood cells and plasma proteins. Nonetheless, reasonable estimates of free aqueous concentrations C_{water} can be obtained using blood or plasma concentrations and taking explicit account of binding. Recommended concentrations for three intravenous agents at physiological pH are given in the table.

For mammals, dose-response curves are exceptionally steep for the induction of general anaesthesia (Fig. 1), so that a concentration only 20% higher than the EC_{50} is sufficient to anaesthetize almost all animals. Moreover, at least for inhalational agents, concentrations only 2 to 4 times higher than the EC_{50} cause deleterious side effects. Thus one should treat with caution experimental data obtained at concentrations in excess of twice the EC_{50} .

EC ₅₀ concentrations for general anaesthesia* in mammals	
Anaesthetic agent	C _{water} (μM)
Halothane†	250
Isoflurane†	320
Enflurane†	620
Methoxyflurane†	330
Chloroform†	1,000
Thiopental‡	25
Pentobarbital§	50
Propofol	0.4

* Lack of response to a painful stimulus.

† For the inhalational agents, C_{water} is an average value⁹ for general anaesthesia in mammals.

‡ Fifty per cent of patients do not respond to a painful trapezius muscle squeeze (a stimulus shown¹¹³ to be equivalent to a surgical incision) at a plasma¹¹³ or serum¹¹⁴ thiopental concentration of ~40 $\mu\text{g ml}^{-1}$. Using a molecular mass $M_r = 241$, together with a value for protein binding^{113,115} of 85%, gives a free aqueous concentration of 25 μM .

§ On recovery of the righting reflex, the plasma concentration of pentobarbital in mice¹¹⁶ is 15.4 $\mu\text{g ml}^{-1}$. Taking a value for plasma protein binding¹¹⁷ of 61% and $M_r = 225$ gives a free aqueous concentration of 27 μM . But anaesthetic EC_{50} concentrations on waking or for abolishing the righting reflex are typically about half those needed to prevent movement in response to a painful stimulus^{114,118}, so that $C_{\text{water}} \approx 50 \mu\text{M}$.

|| On waking, the blood concentration of propofol in patients¹¹⁹ is ~1 $\mu\text{g ml}^{-1}$. Taking a blood/plasma partition coefficient¹²⁰ of 1.3, a value for plasma protein binding¹²⁰ of 97.8% and $M_r = 178$, gives a free aqueous concentration of 0.1 μM . This is comparable to values of 0.15 μM and 0.3 μM that can be calculated from corresponding data¹²¹ for rats and dogs, respectively. Taking an average and (as above) multiplying by two gives $C_{\text{water}} \approx 0.4 \mu\text{M}$.

(Box 2). Interestingly, synaptically evoked postsynaptic currents through putative nAChRs in identified *Aplysia* neurons²⁷ are as sensitive to enflurane as currents evoked by ionophoretically applied ACh, suggesting that the inhibition, at least at these synapses, can be accounted for entirely by postsynaptic (rather than presynaptic) effects.

Many intravenous agents have been shown to increase the decay rate of miniature endplate currents, although rather high concentrations are required for substantial effects³⁰. Other studies have shown that nAChRs can be very sensitive to some, but not all, barbiturates. For example, pentobarbital at low concentrations ($IC_{50} \approx 20 \mu M$) reduces single channel mean open time in denervated rat skeletal muscle³¹ and inhibits agonist-induced cation fluxes in *Torpedo* electroplaque membranes³². However, barbiturate binding to the *Torpedo* nAChR correlates poorly with anaesthetic potencies³³. Also, the *R*(+) isomer of pentobarbital is more effective than the *S*(-) isomer³² at inhibiting the nAChR, in contrast to their potencies in animals (Box 2). Short-chain alcohols are capable of either potentiating or inhibiting the response^{34,35} to ACh (depending upon both alcohol and ACh concentrations), but long-chain alcohols are always

inhibitory^{34,36}. It seems likely that these effects are mediated by the alcohols binding directly to the receptor channel protein (at either excitatory or inhibitory sites), although the extent to which these alcohol-binding sites overlap with those of other anaesthetics has yet to be determined.

Although nicotinic ACh receptors are sensitive to a number of different anaesthetics, it is too early to evaluate whether or not their inhibition contributes to general anaesthesia. This should become clearer when their role in the CNS is better understood.

GABA_A receptors. General anaesthetics might act by potentiating inhibitory synaptic transmission, and the GABA_A receptor channel has long been considered a potential target. As with most popular ideas, however, complications and apparent contradictions appeared as work progressed, and the role of GABA (γ -aminobutyric acid) in anaesthesia has been controversial. Nonetheless, the GABA_A receptor has been established as a prime anaesthetic target by recent electrophysiological studies.

Most anaesthetics are very effective at potentiating responses to GABA. For example, 20 μM pentobarbital increases peak GABA responses by about threefold in *Xenopus* oocytes expressing whole brain messenger RNA, with the effect being accounted for by an apparent increase in receptor affinity for GABA³⁷. (Pentobarbital also speeds up desensitization, but this seems simply to reflect the extent to which the channels are functionally active; that is, it is a secondary consequence of the increased affinity to GABA.) Because the major action of pentobarbital and most other anaesthetics is to shift the GABA dose-response curve to lower concentrations (Fig. 2a), peak Cl^- currents elicited by high levels of GABA are virtually unaffected. Effects on spontaneous inhibitory postsynaptic currents reflect more physiologically relevant interactions between anaesthetics and the postsynaptic receptor, with the decay of the currents being greatly prolonged by pentobarbital, but the rise time and peak amplitude being usually unaffected^{38,39}. This behaviour is consistent with anaesthetics enhancing, and hence prolonging, receptor binding of GABA released presynaptically from vesicles in brief but high concentration pulses. Interesting, the *S*(-) enantiomer of pentobarbital is more effective than the *R*(+) isomer at enhancing GABA-induced conductance in spinal neurons⁴⁰, in line with their relative potencies in animals (Box 2).

A similar picture is emerging for other anaesthetics. The inhalational agents halothane, enflurane and isoflurane all markedly enhance currents induced by low GABA concentrations (Fig. 2b, c), but are ineffective (or even slightly inhibitory) at high GABA concentrations^{20,41,43}. As with the barbiturates³⁷, the potentiation can be explained most simply by an anaesthetic-induced increase in the apparent affinity of GABA^{20,44}. Stereoselectivity has been observed with an inhalational agent; the *S*(+) enantiomer of isoflurane, which is most potent in animals (Box 2), is about twice as effective as the *R*(-) isomer at prolonging evoked inhibitory postsynaptic currents (i.p.s.cs) mediated by GABA_A receptor channels in cultured rat hippocampal neurons⁴⁵. The residual steady-state current following desensitization by high levels of GABA is inhibited rather than enhanced by volatile agents⁴¹, although the relevance of this to fast synaptic events is uncertain. The aliphatic alcohols appear to behave like the volatile anaesthetics⁴⁶. In addition, the intravenous anaesthetics propofol⁴⁷ and alphaxalone^{48,49} enhance the action of GABA in a variety of systems. For the steroid anaesthetic alphaxalone, the potentiation is stereoselective, with the non-anaesthetic 3- β -hydroxy-isomer, betaxalone, being ineffective⁴⁹.

Single channel studies with volatile agents⁵⁰, barbiturates⁵¹, propofol⁴⁷ and anaesthetic steroids⁵² show no changes in channel conductance, but the channel open time increases (for example, see Fig. 2d), consistent with the prolongation of postsynaptic currents (Fig. 2e). Thus the molecular basis for the potentiation of GABA action by anaesthetics is an increased open probability for the GABA_A receptor channel; the extent to which this is due

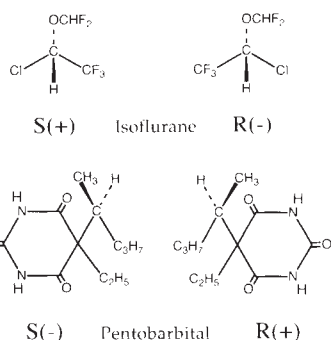
BOX 2 Is general anaesthesia stereoselective?

GENERAL anaesthesia can be induced by a remarkable variety of chemical agents, including simple alcohols, alkanes, ketones, ethers and even some inert gases. Clearly no specific chemical group is required for activity. But this background of nonspecificity has tended to obscure the fact that most clinically useful agents are more complex compounds; in particular, their structures generally include an asymmetric carbon atom, so that the anaesthetic can exist in two enantiomeric forms (see figure). In practice, such agents are almost invariably administered as racemic mixtures, but when the potencies of individual optical isomers have been tested on mammals, they are stereoselective, with one of the isomers being more potent.

Stereoselectivity was first observed with the barbiturates. The optical isomers of thiopental, pentobarbital, secobarbital and hexobarbital, for example, differ in their potencies in mammals by ~2-fold^{122,123}. In general, for the barbiturates, the *S* isomer is more potent than the *R* isomer. The two optical isomers of the dissociative anaesthetic ketamine also differ in their anaesthetic properties, with the *S*(+) isomer being 2 to 4 times more potent than the *R*(-) isomer in both mice¹²⁴ and humans¹²⁵. Although with any intravenous agent it is difficult to assess accurately the extent to which pharmacokinetic factors (rates of metabolism, permeabilities, plasma protein binding, and so on) influence measured differences in potencies, the available evidence indicates that the observed stereoselectivity of intravenous agents is a real reflection of differences in their intrinsic potencies^{122-124,126,127}.

Optical isomers of the inhalational anaesthetic isoflurane, which are not significantly metabolized, are now available. When tested⁵⁹ for their anaesthetic effects in mice, the *S*(+) isomer was ~50% more potent than the *R*(-) isomer. In these experiments⁵⁹, sleep times were recorded following intraperitoneal injection, so that confirmation of this stereoselectivity will require a determination of anaesthetic EC_{50} concentrations.

The clear picture that emerges from anaesthetic potency measurements in mammals is that many general anaesthetics are stereoselective in their actions. Because the potency ratios observed with certain *in vitro* targets are comparable (see text) to those *in vivo*, anaesthetics probably exert their primary effects at a relatively small number of sites. Stereoselectivity may prove to be one of the most powerful guides as to which *in vitro* targets are relevant to anaesthesia.



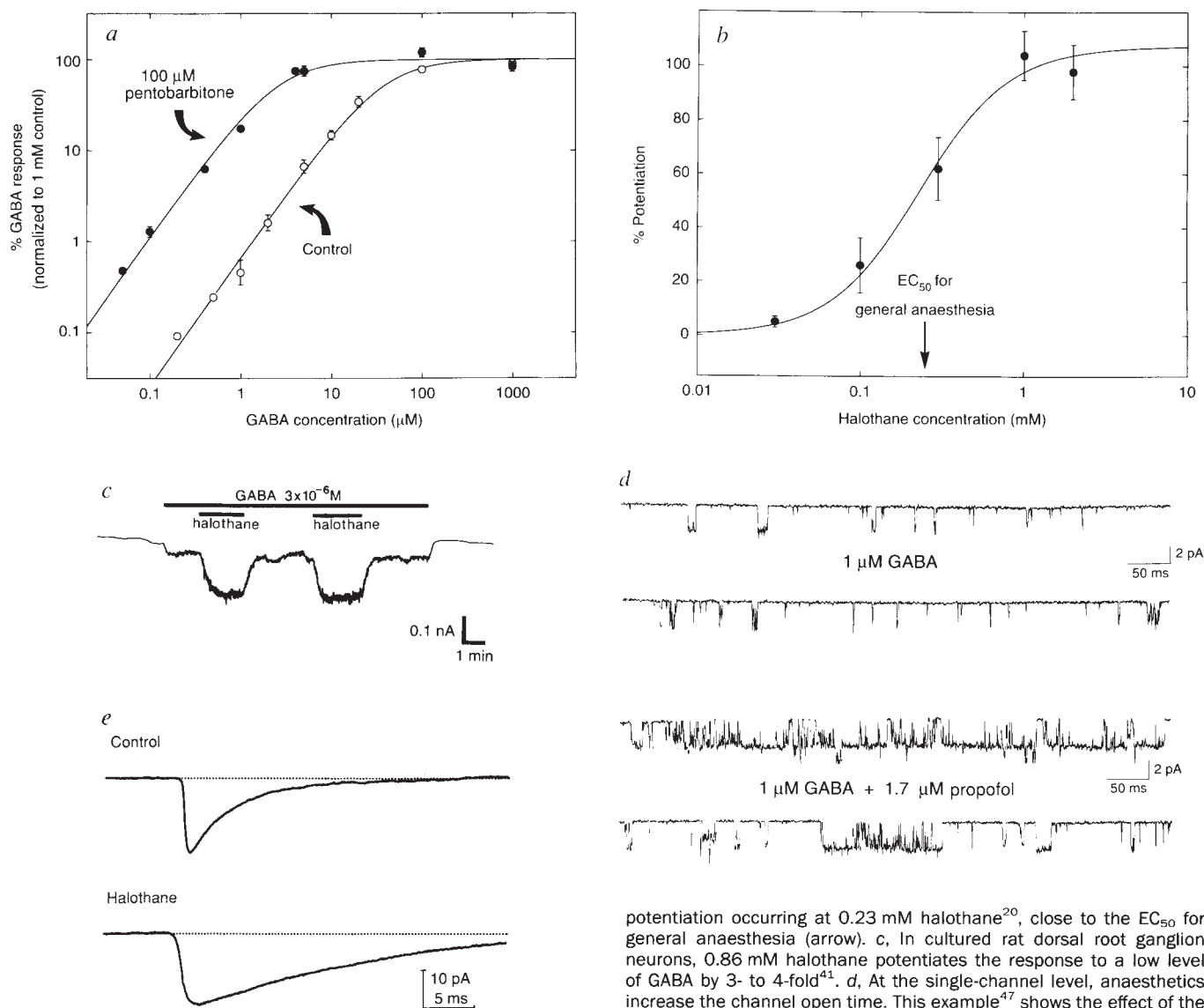


FIG. 2 General anaesthetics potentiate the actions of GABA on the GABA_A receptor-channel complex. This is illustrated by recent electrophysiological results. *a*, 100 μ M pentobarbital greatly enhances GABA-induced Cl^- currents in *Xenopus* oocytes injected with rat brain mRNA at low GABA concentrations but has little effect at high GABA concentrations, reflecting an increased apparent affinity of GABA for its receptor³⁷. *b*, Clinically relevant concentrations of halothane potentiate responses to low levels of GABA (3 μ M) in dissociated rat brain neurons, with 50%

potentiation occurring at 0.23 mM halothane²⁰, close to the EC_{50} for general anaesthesia (arrow). *c*, In cultured rat dorsal root ganglion neurons, 0.86 mM halothane potentiates the response to a low level of GABA by 3- to 4-fold⁴¹. *d*, At the single-channel level, anaesthetics increase the channel open time. This example⁴⁷ shows the effect of the intravenous anaesthetic propofol on GABA_A channels in bovine chromaffin cells. The probability that a channel is in a conducting state is increased by ~4-fold by 1.7 μ M propofol. *e*, At a functional synapse, GABA is released presynaptically as a transient but very high concentration pulse. Under voltage-clamp conditions, the peak height of the spontaneous inhibitory postsynaptic current (i.p.s.c.) remains unchanged but the time course of the decay is prolonged by anaesthetics. This example³⁹ shows averages of 100 spontaneous i.p.s.c.s recorded from rat hippocampal neurons in the presence and absence of 0.40 mM halothane.

to enhanced GABA binding, or to changes in channel gating, remains to be determined and may depend on the anaesthetic.

Generally speaking, almost all agents (ketamine may be an exception) at their animal half-maximal effective concentrations EC_{50} (Box 1) enhance the current induced by low levels of GABA by over 50%. (Note that a 50% increase in an inhibitory conductance can affect the membrane potential as much as a 50% inhibition of an excitatory conductance.) Does this remarkably consistent picture of potentiation of postsynaptic currents, however, translate into an enhancement of inhibitory synaptic transmission? Surprisingly little unambiguous evidence is available to answer this key question, although clear examples of synaptic potentiation (such as prolongation of evoked postsynaptic currents) have been reported^{48,53}. On the other hand, some (presumably) GABAergic synapses appear to be inhibited^{6,54}. Some of these discrepancies may only be apparent. For example, experi-

mental observation of inhibitory synaptic activity often requires activation of local interneurons by excitatory synapses, which may themselves be depressed by anaesthetics. Other discrepancies are probably real. The degree of anaesthetic potentiation of GABA_A receptors, for example, depends upon their subunit compositions⁴⁴, and the distribution of subunits throughout the CNS varies greatly. Indeed, the effects of anaesthetic steroids on GABA_A receptors vary considerably between different brain regions⁵⁵. In addition, anaesthetic responses may be modulated by second messenger systems, which can vary from cell to cell. The potentiating effect of ethanol apparently requires a particular γ -subunit that contains a short sequence of amino acids with a phosphorylation site for protein kinase C⁵⁶. Also, intracellular levels of Ca^{2+} may³⁹ or may not^{43,45} influence the anaesthetic response.

Although uncertainties remain as to the extent to which intact

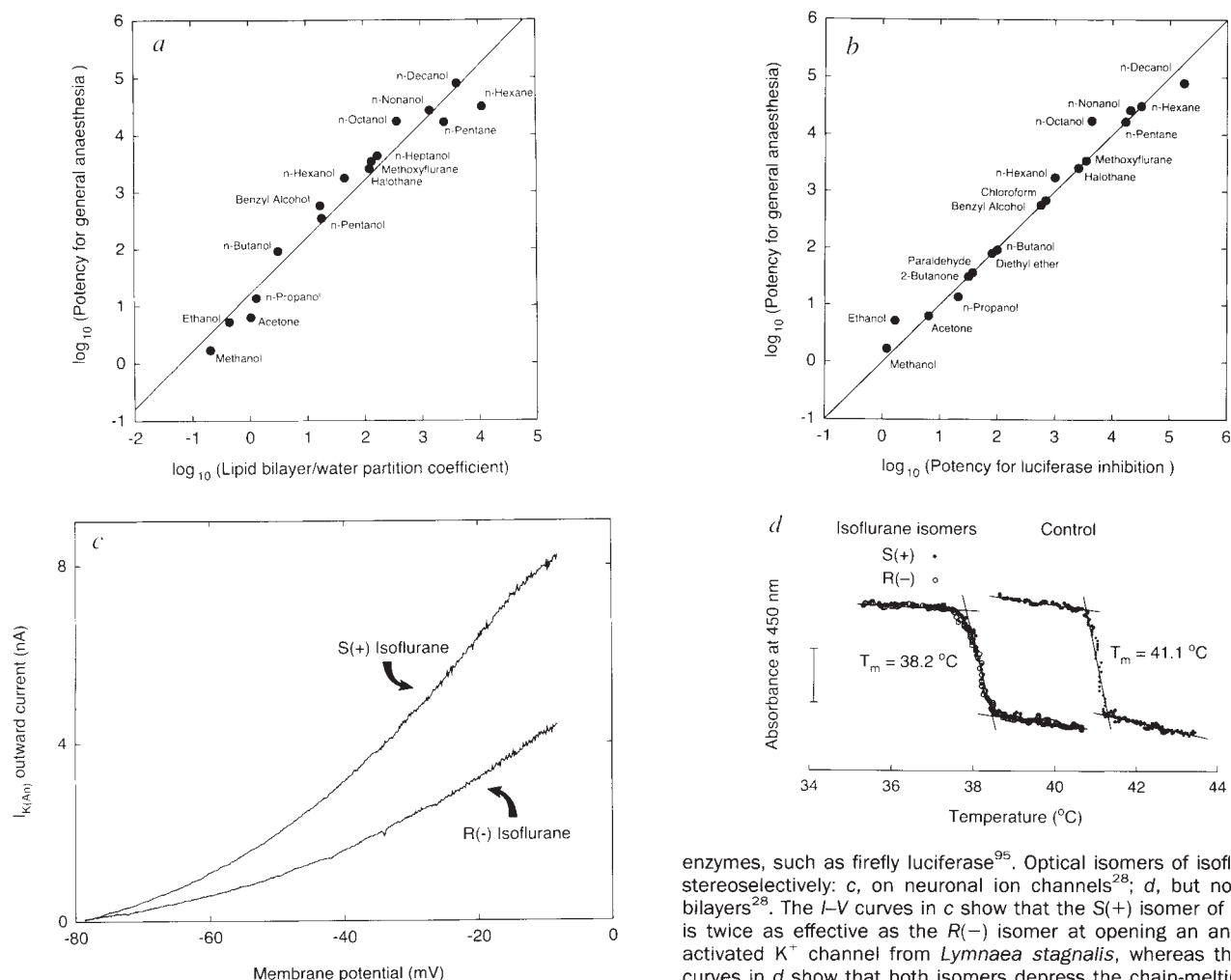


FIG. 3 General anaesthetics act by binding directly to proteins. *a*, The Meyer–Overton correlation has traditionally been interpreted as meaning that the primary target sites are lipid portions of nerve membranes^{87,88}. In its modern form, shown here, a good correlation is seen to exist between the potency of an anaesthetic (reciprocal of its molar EC₅₀ concentration for anaesthesia) and its lipid/water partition coefficient. *b*, General anaesthetic potencies in animals can be correlated equally well with their ability to inhibit the activity of certain soluble

enzymes, such as firefly luciferase⁹⁵. Optical isomers of isoflurane act stereoselectively: *c*, on neuronal ion channels²⁸; *d*, but not on lipid bilayers²⁸. The *I*–*V* curves in *c* show that the S(+) isomer of isoflurane is twice as effective as the R(–) isomer at opening an anaesthetic-activated K⁺ channel from *Lymnaea stagnalis*, whereas the melting curves in *d* show that both isomers depress the chain-melting phase-transition temperature (T_m) of a pure lipid bilayer (a measure of bilayer disruption) by the same amount. The general anaesthetic potencies in *a* and *b* are taken from refs 9 and 100, or those cited in ref. 95. The lipid bilayer partition coefficients are from refs cited in ref. 87, except for the values for pentane, hexane and decanol, which were extrapolated from values for lower members of the homologous series. The luciferase inhibition data in *b* are from ref. 95; the data in *c* and *d* are from ref. 28. The line in *a* is the least-squares line of unit slope; the line in *b* is the line of identity. The calibration bar in *d* represents a change in absorbance of 0.04 units.

GABAergic synapses are functionally potentiated, there is now an impressive body of evidence that points to the GABA_A receptor channel complex as a major target for most general anaesthetics.

Other inhibitory ion channels. GABA is the most important inhibitory neurotransmitter in the brain, but glycine plays a major role in the spinal cord and lower brainstem. The possible importance of these lower CNS areas, and hence of glycine, in general anaesthesia is emphasized by a recent decerebration experiment⁵⁷, which found the minimum alveolar concentration of isoflurane to be independent of cortical or forebrain structures in the rat. Nonetheless, no comprehensive anaesthetic studies have been done on the glycine receptor channel. One investigation²⁰ with dissociated neurons from the rat nucleus tractus solitarius found that relevant levels of volatile agents substantially potentiate inhibitory Cl[–] currents activated by low but not high glycine concentrations, reflecting (as with the GABA_A receptor) an apparent increase in agonist affinity. In addition, a study⁴⁷ on spinal neurons found considerable potentiation by propofol but not pentobarbital (although a fairly high glycine concentration was used).

An inhibitory synaptic K⁺ current, $I_{K(AN)}$, activated by low levels of volatile agents, has been found^{4,28,58} in molluscan neurons. It is present in certain anaesthetic-sensitive pacemaker neurons but absent from insensitive neurons. $I_{K(AN)}$ is rapidly and reversibly activated by anaesthetics, does not inactivate with time, is not voltage-gated, and responds stereoselectively to the optical isomers of isoflurane (Fig. 3c) with the same order of potency as general anaesthesia in mice⁵⁹. Although these properties make it an attractive candidate for a target in general anaesthesia, it has yet to be determined whether $I_{K(AN)}$ is found in mammalian neurons.

Inhibitory synapses are also attractive candidates for explaining the well-known pressure reversal of general anaesthesia. For example, the effects of pressure resemble those of strychnine⁶⁰, a glycine antagonist, and pressure reversal is not observed⁶¹ with shrimps (which are thought not to use glycine as a neurotransmitter). As pressure almost invariably depresses synaptic transmission *in vitro*^{62,63}, pressure reversal could, in principle, be explained by pressure blocking inhibitory synapses, but there is now much less conviction that pressure reversal will provide fundamental clues as to how anaesthetics act. The phenomenon

of pressure reversal may prove to be incidental, rather than central, to mechanisms of general anaesthesia^{63, 65}.

Presynaptic versus postsynaptic targets

Although it is clear that anaesthetics can have substantial effects on postsynaptic membranes, the extent to which they also act presynaptically is difficult to assess. One attractive proposal⁶⁶ was that anaesthetics might act by discharging pH gradients across the membranes of synaptic vesicles, resulting in leakage of catecholamines, but subsequent measurements⁶⁷ have shown the effects to be small. There have been many attempts to measure neurotransmitter release directly in brain slices following electrical⁶⁸ or K⁺-induced^{69–71} stimulation. The results vary with both preparation and neurotransmitter and often show small, but significant, changes (usually reductions) in the amount of neurotransmitter released because of the presence of anaesthetics. The multiple pathways involved in such preparations, however, make simple molecular interpretations almost impossible. An alternative approach has been to use relatively simple reflex pathways in spinal cord preparations and to disentangle pre- and postsynaptic effects by comparing evoked with spontaneous postsynaptic potentials. The most recent and careful of these studies^{72, 73} suggest that, at relevant concentrations of halothane and thiopental, neurotransmitter release might be inhibited by about 20%. This inhibition is most probably a result of reduced Ca²⁺ entry, rather than an effect on the exocytotic mechanisms underlying vesicle release⁷⁴. Overall, it is likely that some anaesthetic inhibition of transmitter release occurs at many synapses, although the effects are small. However, more work is needed to establish the role presynaptic effects play in the overall anaesthetic sensitivity of synaptic transmission.

Second messenger systems

Second messengers regulate the activity of many enzymes and ion channels, sometimes by direct binding but more often by phosphorylation. One of the most important second messengers is free intracellular Ca²⁺, and it has long been thought that anaesthetics might disrupt neuronal function by increasing its concentration ([Ca²⁺]_i). With the advent of Ca²⁺-sensitive fluorescent dyes, it is now possible to test this idea. Studies on several systems^{75–79} generally show no significant sustained (as opposed to transient) effect on resting [Ca²⁺]_i at relevant anaesthetic levels and inhibition (rather than potentiation) of agonist or K⁺-depolarization-induced increases in [Ca²⁺]_i. The inhibitory effects have been variously ascribed to blocking of voltage-gated or receptor-operated Ca²⁺ channels, inhibition of inositol trisphosphate (InsP₃) production, or depletion of internal Ca²⁺ stores. Regarding InsP₃, which releases Ca²⁺ from internal stores, anaesthetics at relevant levels produce little⁸⁰ or no^{79, 81} inhibition of agonist-induced accumulation of total inositol phosphates (assumed, perhaps erroneously, to reflect InsP₃ levels), although substantial effects have been reported⁷⁸ for halothane in vascular smooth muscle cells. Another branch of the phosphatidylinositol second messenger pathway produces diacylglycerol, an activator of protein kinase C (PKC). The effects of anaesthetics on diacylglycerol production are not known, but its PKC target can be inhibited by anaesthetics. Although PKC inhibition seems to be small^{82, 83}, anaesthetics inhibit a lipid-free form of the enzyme by binding to its regulatory subunit, and the anaesthetic sensitivity of lipid-bound PKC depends on the lipid composition⁸³. Finally, it may be significant that liver cytochrome P450-mediated metabolism of arachidonic acid, an eicosanoid second messenger that can also be derived from inositol phospholipids, is inhibited very sensitively by a wide range of general anaesthetics⁸⁴.

Cyclic nucleotide levels have been measured in the CNS of animals killed after exposure to anaesthetics. A consistent finding is a substantial reduction in cerebellar cGMP⁹, but with barbiturates this occurs at concentrations that do not reduce locomotor activity⁸⁵, suggesting that the effect may be unrelated

to general anaesthesia. Most *in vitro* studies on cyclic nucleotides have used high anaesthetic concentrations, although one⁸¹ showed that 1.25% atm. halothane at 37 °C (free aqueous concentration, C_{water} ≈ 350 μM) had no effect on basal or noradrenaline-induced cAMP accumulation in brain slices. The minimal alveolar concentration of halothane can be greatly reduced by selective α₂-adrenergic agonists⁸⁶, which, among other actions, inhibit adenylyl cyclase. However, there is no reason for believing that halothane acts on the same pathways. Indeed, it is notable that selective α₂-adrenergic antagonists, although they block the halothane-sparing actions of specific α₂-agonists, do not affect the minimal alveolar concentration of halothane on their own⁸⁶.

There is as yet little hard evidence that second messenger systems are involved in general anaesthesia. At high enough concentrations, anaesthetics can substantially affect many second messenger systems (as shown in many reports not included here), but the few studies made at relevant concentrations suggest that they may be relatively insensitive to anaesthetic perturbation.

Molecular nature of anaesthetic target sites

If general anaesthetics act by selectively targeting synaptic ion channels or the systems that regulate them, the question then arises as to how, at the molecular level, they exert their effects. Ever since Meyer and Overton discovered a correlation between anaesthetic potency and partitioning into fat-like solvents⁶⁴ (Fig. 3a), the traditional view has been that general anaesthetics act by disrupting the structure or dynamic properties of the lipid portions of nerve membranes^{87, 88}. By the early 1980s, however, accumulating evidence posed serious quantitative problems for this simple unifying idea^{64, 89}. Most importantly, effects on lipid bilayers at relevant anaesthetic levels were found to be implausibly small: they could generally be mimicked by temperature changes of less than 1 °C. Taken together with positive evidence for the direct involvement of proteins (see later), this has led to the almost complete abandonment of theories that postulate changes in the properties of the bulk lipid bilayer (but see ref. 90). Lipid hypotheses have subsequently been refined^{91–94} and in general postulate that specialized 'domains' in membranes are not only particularly sensitive to anaesthetics, but are critical to membrane function. Probably the best formulated of these is the hypothesis that the boundary lipids surrounding membrane proteins are preferentially affected, but recent work⁹⁴ casts doubt on this idea.

Most drugs whose mechanisms are known act by binding directly to proteins. Strong evidence that general anaesthetics might also act in this way came with the demonstration⁹⁵ that the activity of a soluble lipid-free enzyme, firefly luciferase, could be inhibited by a diverse range of general anaesthetics at IC₅₀ concentrations very close to animal EC₅₀ values (Fig. 3b). These observations provided not only a viable alternative to the lipid hypotheses, but also a simple explanation for the Meyer–Overton correlation. Subsequent studies showed that this enzyme can be transformed allosterically between anaesthetic-sensitive and insensitive forms⁹⁶. Anaesthetic-binding sites that mimic the Meyer–Overton correlation have also been found on other enzymes^{83, 97}, whereas some proteins⁹⁸ are sensitive only to certain anaesthetics. Finally, in a recent photoaffinity labelling study⁹⁹, saturable binding of halothane (K_d = 490 μM) to rat brain synaptosomes (presumably to proteins) has been reported.

Exceptions often prove more instructive than rules. Because both binding to proteins and dissolving in lipids can account for the Meyer–Overton correlation, attention has focused on the curious lack of anaesthetic potency of long-chain compounds. In the best-studied series, the *n*-alcohols, a cut-off occurs for tadpoles after dodecanol¹⁰⁰ (that is, a saturated solution of tri-decanol does not cause anaesthesia), and for the *n*-alkanes the cut-off in mice is after octane¹⁰¹. With lipid-free enzymes, similar cut-offs are observed^{96, 98, 102}, with the exact points of cut-off being somewhat variable. These cut-offs are most simply explained by

anaesthetics binding to protein pockets or clefts with circumscribed dimensions¹⁰². Explanations in terms of lipid theories have been more problematical. It was once suggested¹⁰³ that long-chain alcohols were simply not sufficiently soluble in bilayers, but this has since been shown to be incorrect¹⁰⁴. More recently, it was found that whereas anaesthetic alcohols can disorder the lipids of synaptic membranes, non-anaesthetic alcohols above a certain chain-length actually increase the order; it was argued that these changes in membrane perturbation might account for the cut-off effect¹⁰⁵. A prediction of this model is that compounds much larger than the cut-off should be anaesthetic antagonists. This has yet to be tested, but compounds just above the cut-off appear to be partial anaesthetics^{101,106}.

Probably the most definitive evidence that general anaesthetics act by binding directly to proteins comes from observations of stereoselectivity (Box 2). Even the relatively simple inhalational agent isoflurane has now been shown to act stereoselectively, not only on mammals⁵⁹ but also on neuronal ion channels^{28,45} (Fig. 3c). Isoflurane does not, however, show stereoselective effects on pure lipid bilayers²⁸ (Fig. 3d), although additional data are needed on how the isoflurane enantiomers interact with cholesterol-containing bilayers.

Taken together, the available evidence strongly suggests that general anaesthetics act by binding directly to proteins. Why, then, are some proteins sensitive to anaesthetics whereas others (probably the majority) are not, and what is the nature of the binding sites on sensitive proteins? Some workers have argued that hydrogen bonding has a crucial role^{107,110}, and there is evidence that, on average, anaesthetic target sites are better hydrogen-bond acceptors than donors¹¹¹. Whether this has any mechanistic significance is not yet clear. We think it likely that the relevant binding sites are hydrophobic pockets exposed to water rather than interfacial sites exposed to lipid hydrocarbon chains⁸⁹. This is because the former, water-filled pockets might be expected to bind hydrophobic anaesthetics more tightly (avoiding competition from lipid hydrocarbon chains); in addition, they can more easily account for the cut-off effect⁸⁹. There is as yet, however, no definitive evidence as to the location of anaesthetic binding sites on membrane proteins (extracellular, intracellular or transmembrane). Finally, the unusually large enthalpy changes observed when anaesthetics bind to firefly luciferase¹¹² may provide a clue as to what makes this protein so sensitive, perhaps reflecting anaesthetic-induced allosteric conformational changes⁹⁶ similar to those that must occur in the GABA_A receptor channel.

Overview

Although general anaesthetics at high enough levels can act non-specifically on a wide variety of neuronal sites, at clinical concentrations they are much more selective and probably exert their primary effects at a relatively small number of CNS targets. These are most likely to be postsynaptic ligand-gated ion channels. Some agents act predominantly at excitatory receptors (such as ketamine at NMDA receptors), but anaesthetic potentiation of inhibitory synaptic receptors (mainly GABA_A) best matches the pharmacological profile of a wide variety of agents for producing general anaesthesia in mammals. Some uncertainty remains, however, over the extent to which the activity of intact inhibitory synapses is potentiated by anaesthetics. In addition, some agents (pentobarbital for example) are clearly effective at both inhibitory and excitatory postsynaptic receptors, so the balance between the inhibition of excitatory synapses and the potentiation of inhibitory synapses in causing general anaesthesia still needs to be established.

Although much attention has focused on voltage-gated ion channels, as a class they are very resistant to clinical concentrations of general anaesthetics. Some voltage-gated Ca²⁺ channels are somewhat more sensitive, and their inhibition may underlie the small reduction of neurotransmitter release that occurs at some synapses.

At the cellular level, anaesthetic actions vary from neuron to neuron owing to the presence of differing ion channels and ion-channel subunits, as well as second messenger regulatory systems. But the case for any major involvement of second messenger systems in general anaesthesia is unproven; the few studies at pharmacologically relevant concentrations indicate that they may be relatively insensitive to anaesthetics, but further investigation should provide more definitive evidence.

At the molecular level, anaesthetics almost certainly act by binding directly to proteins rather than by perturbing lipid bilayers. Anaesthetics probably influence protein activity by binding to pockets or clefts and causing small changes in protein conformation. □

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ARTICLES

Atomic model of plant light-harvesting complex by electron crystallography

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The structure of the light-harvesting chlorophyll *a/b*-protein complex, an integral membrane protein, has been determined at 3.4 Å resolution by electron crystallography of two-dimensional crystals. Two of the three membrane-spanning α -helices are held together by ion pairs formed by charged residues that also serve as chlorophyll ligands. In the centre of the complex, chlorophyll *a* is in close contact with chlorophyll *b* for rapid energy transfer, and with two carotenoids that prevent the formation of toxic singlet oxygen.

PHOTOSYNTHESIS in all green plants uses solar energy collected by the light-harvesting chlorophyll *a/b*-protein complex associated with photosystem II (LHC-II). LHC-II is the most abundant membrane protein in chloroplasts, accounting for half of the pigments involved in plant photosynthesis. Each polypeptide of 232 amino acids^{1,2} binds and organizes a minimum of 12 chlorophylls and 2 carotenoids (xanthophylls) for light-harvesting and energy transfer to the reaction centres of photosystems I and II.

The molecular mechanisms underlying these processes can be understood only if the structures of the participating pigment-protein complexes in the photosynthetic membrane are known in detail. The structure of a bacterial photosynthetic reaction centre has been solved at high resolution^{3,4}, and that of a cyanobacterial photosystem I complex has been solved at 6 Å (ref. 5). So far, the most detailed structure of a membrane antenna complex has been a 6 Å map of LHC-II (ref. 6). Here we present an

atomic model of LHC-II based on a three-dimensional map at 3.4 Å resolution.

High-resolution electron microscopy

Detergent-solubilized, purified LHC-II forms highly ordered two-dimensional crystals^{7,8}, which are ideal objects for electron crystallography. Electron diffraction of two-dimensional crystals yielded the structure factor amplitudes⁹. Phases were determined directly by processing^{10–12} electron micrographs of tilted two-dimensional crystals. Images were recorded with an electron cryomicroscope capable of resolving 2.0 Å object features at a specimen-stage temperature of 4.2 K (ref. 13). At very low temperature the effects of radiation damage are much less severe than at room temperature. More electrons can therefore be used to take an image, so that high-resolution phases can be determined more accurately.

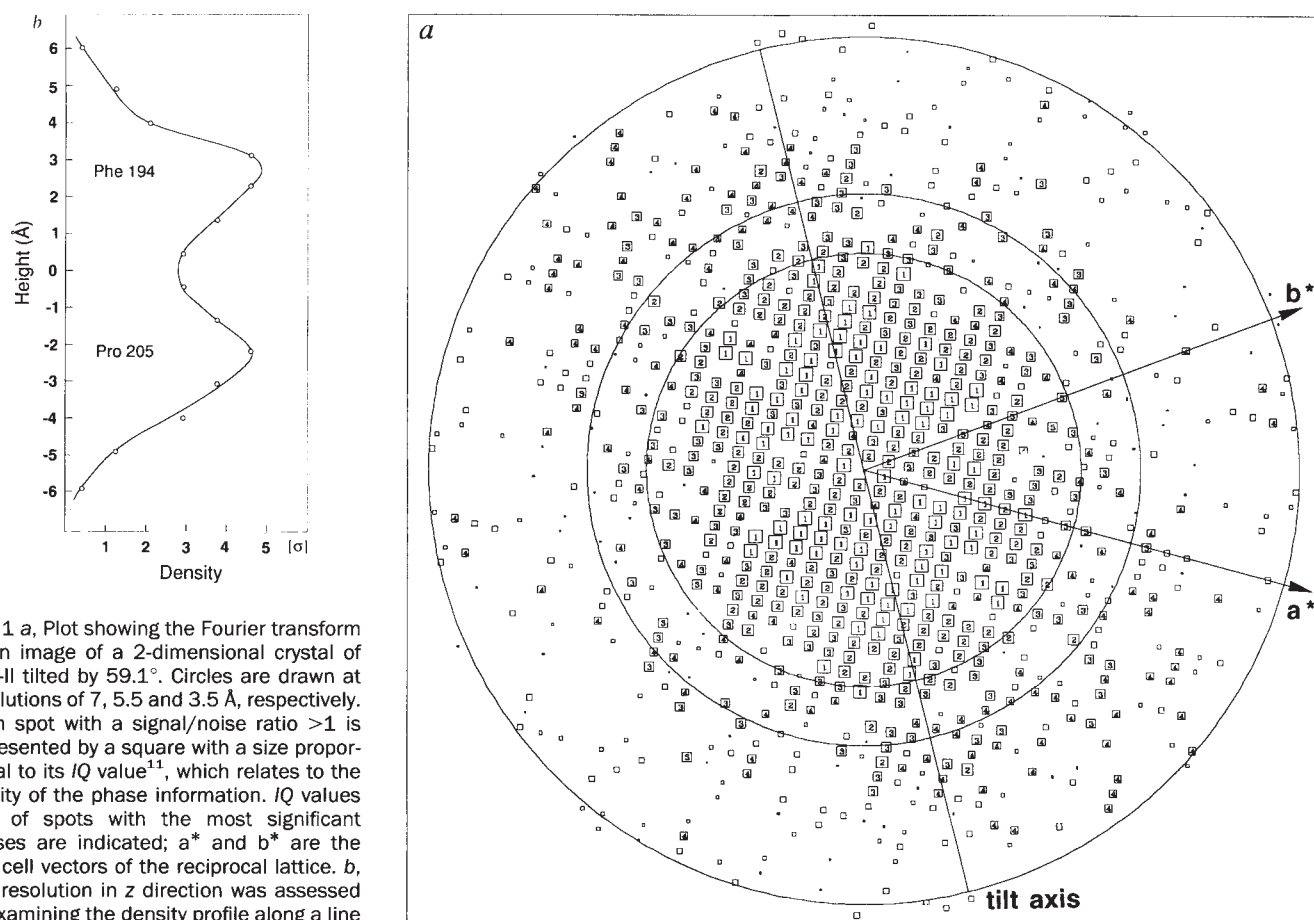


FIG. 1 a, Plot showing the Fourier transform of an image of a 2-dimensional crystal of LHC-II tilted by 59.1° . Circles are drawn at resolutions of 7, 5.5 and 3.5 Å, respectively. Each spot with a signal/noise ratio >1 is represented by a square with a size proportional to its IQ value¹¹, which relates to the quality of the phase information. IQ values 1–4 of spots with the most significant phases are indicated; a^* and b^* are the unit cell vectors of the reciprocal lattice. b, The resolution in z direction was assessed by examining the density profile along a line perpendicular to the membrane plane through the side-chain densities of Phe 194 and Pro 205 in the 3-dimensional map. The phenyl ring of Phe 194 is almost exactly above the ring of Pro 205 (Fig. 2e), with a vertical distance between the ring centres of 4.9 Å. According to the Rayleigh criterion⁴⁶, two points are considered to be just resolved if the saddle-to-peak intensity ratio is 0.81. In our case, this ratio is 0.6, indicating an effective resolution in the z direction of better than 4.9 Å. At $(4.9 \text{ Å})^{-1}$ in z^* and a maximum tilt angle of 60° , 75% of the Fourier components extending to $(3.4 \text{ Å})^{-1}$ are still sampled.

METHODS. Two-dimensional crystals of LHC-II were grown as described⁸. Specimens for electron microscopy were prepared⁸ on molybdenum grids⁴⁷ with laminar carbon support film⁴⁸. Electron diffraction patterns were recorded and processed and electron diffraction amplitudes were merged as described⁹. Phases were obtained from high-resolution micrographs taken at tilt angles ranging from 0 – 60° in a JEOL4000 SFX electron cryomicroscope¹³. Specimens washed with 0.7% tannin⁸ were transferred with a specially designed cryo-transfer system into the top entry stage¹³ precooled with superfluid helium to 1.5 K. The specimen temperature was raised to 4.2 K to prevent stage vibrations caused by turbulent flow of liquid helium. Flood-beam images

were recorded at a temperature below 20 K with an electron dose of 25 – 35 e Å^{-2} , using a minimum-dose system⁴⁹. Micrographs were recorded on Kodak SO163 electron emulsion film at a magnification of $60,000\times$ with an accelerating voltage of 400 kV and 2 s exposure. Films were developed to an optical density of ~ 0.7 in full-strength Kodak D-19 developer. Images were processed according to refs 11 and 12, with programs provided by R. Henderson. Briefly, micrographs of highly ordered 2-dimensional crystals were selected by optical diffraction and areas of $6,000 \times 6,000$ image points were digitized on a Perkin-Elmer 1010 GM microdensitometer with a circular aperture of $10 \mu\text{m}$ at a stepsize of $7 \mu\text{m}$, corresponding to 1.17 Å at the specimen. Computer transforms of images corrected for small distortions of the 2-dimensional crystal lattice were generated and corrected for contrast transfer, objective lens astigmatism, beam tilt, and the gradient of defocus across the processed area^{11,12}. Amplitudes and phases were combined iteratively¹² and sampled at a distance corresponding to more than twice the thickness of the 2-dimensional crystals to follow the variation of structure factors accurately.

A three-dimensional map of LHC-II was calculated, using amplitude and phase data (Fig. 1) to 3.4 Å resolution. Because it is difficult to record images and diffraction patterns at tilt angles above 60° , there is a conical region in reciprocal space in which amplitudes and phases cannot be measured (Table 1). Model calculations indicate that at 60° tilt, the resolution in the direction perpendicular to the membrane plane is worse by a factor of about 1.3 (ref. 14) compared with the in-plane resolution. The effective resolution of the map in z direction may not reach this limit but is better than 4.9 Å , as indicated by the density profile of two amino-acid side chains separated vertically by this distance (Fig. 1b). The anisotropy of resolution did not impair our ability to trace the polypeptide and to distinguish the bound pigment molecules owing to the high quality of the phase data (Table 1), which means that the noise level of the map is

low. With 12 chlorophylls, 2 carotenoids and about 80% of the polypeptide (residues 26 to 100 and 116 to 224) fitted, a crystallographic R -factor of 33.0% and a free R -factor of 37.9% were calculated using phase and amplitude constraints¹⁵. The starting R -factor was 45%.

Transmembrane helices

The three transmembrane α -helices are referred to as B, C, A, as before⁶. Bulky side-chain densities provided guide points for an unambiguous fit of the peptide chain in the membrane-spanning regions. These residues include Arg 70, Trp 71, Met 73 in the first transmembrane helix in the sequence (helix B; Fig. 2a) and Arg 185, Leu 186, Met 188, Phe 192, Phe 194 and Phe 195 in the third transmembrane helix (helix A; Fig. 2b). Helix B is 51 Å long, extending from Pro 55 to Gly 89 in 9.5 turns. Helix

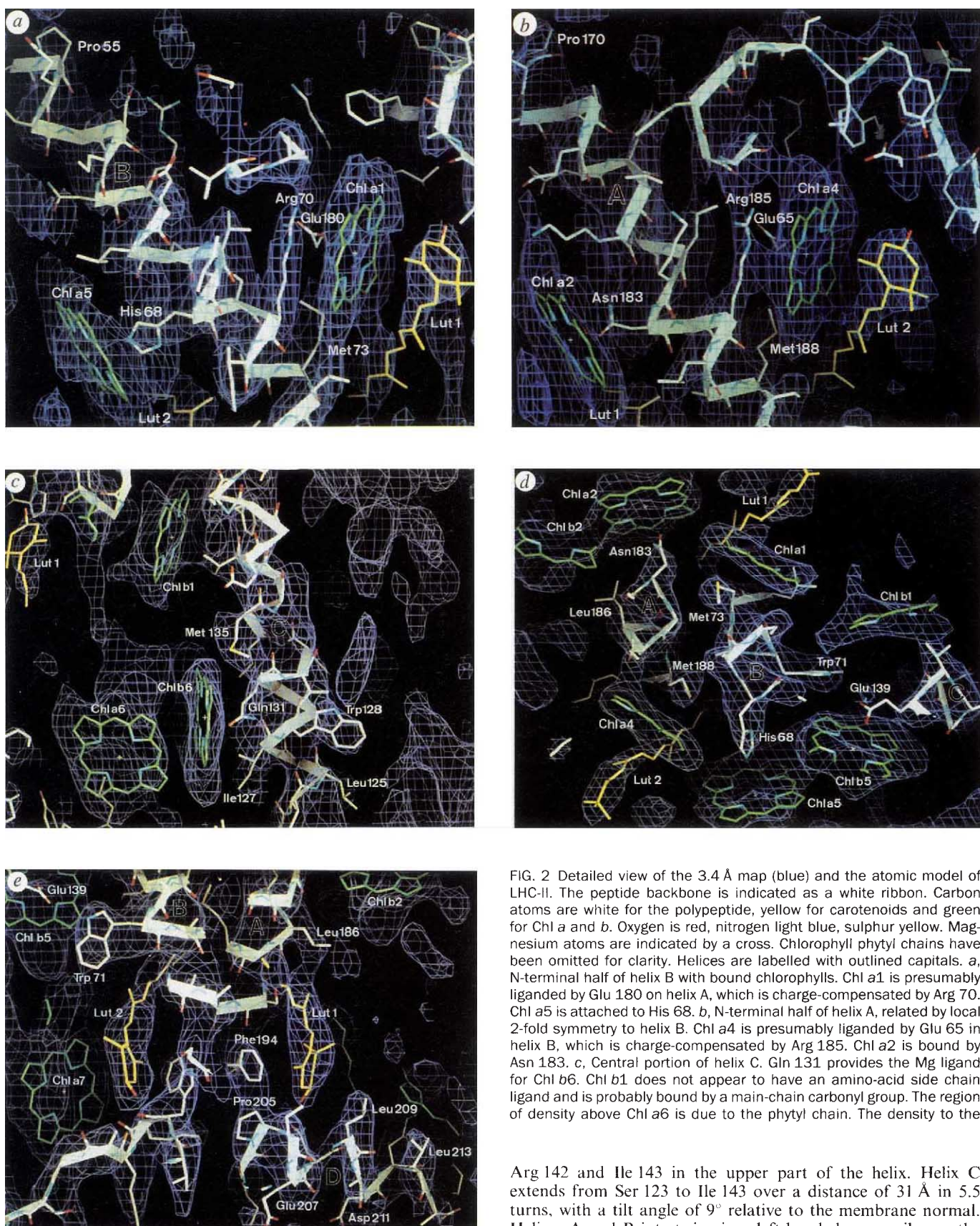
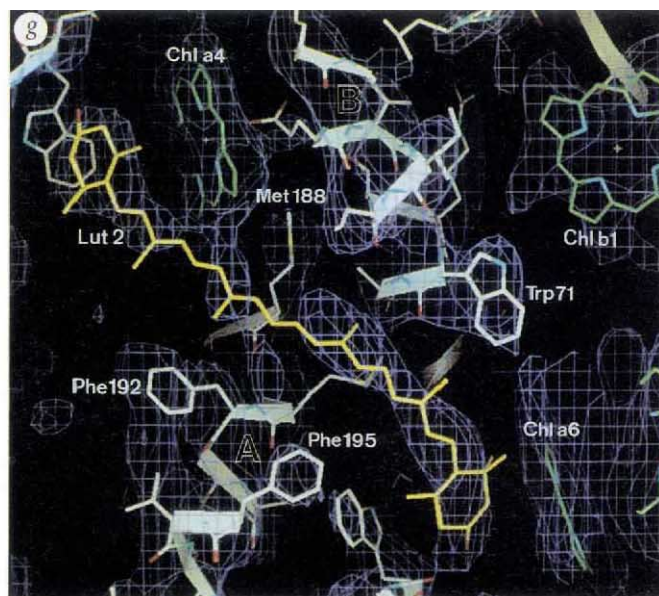
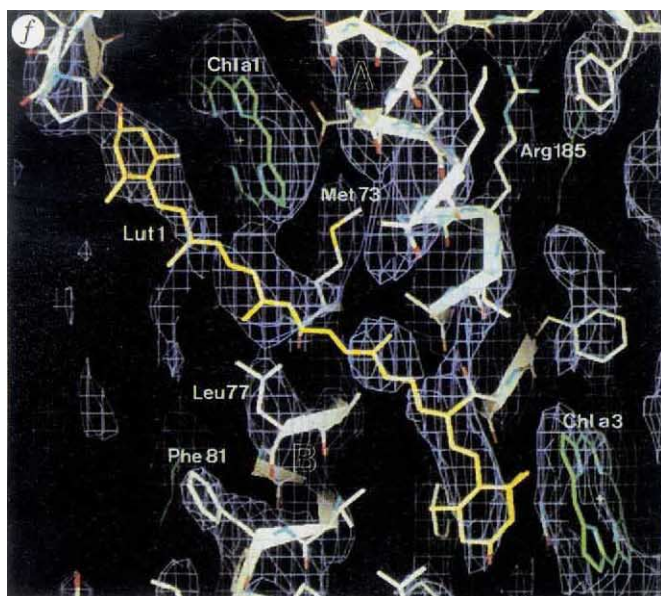


FIG. 2 Detailed view of the 3.4 Å map (blue) and the atomic model of LHC-II. The peptide backbone is indicated as a white ribbon. Carbon atoms are white for the polypeptide, yellow for carotenoids and green for Chl a and b. Oxygen is red, nitrogen light blue, sulphur yellow. Magnesium atoms are indicated by a cross. Chlorophyll phytol chains have been omitted for clarity. Helices are labelled with outlined capitals. **a**, N-terminal half of helix B with bound chlorophylls. Chl a1 is presumably liganded by Glu 180 on helix A, which is charge-compensated by Arg 70. Chl a5 is attached to His 68. **b**, N-terminal half of helix A, related by local 2-fold symmetry to helix B. Chl a4 is presumably liganded by Glu 65 in helix B, which is charge-compensated by Arg 185. Chl a2 is bound by Asn 183. **c**, Central portion of helix C. Gln 131 provides the Mg ligand for Chl b6. Chl b1 does not appear to have an amino-acid side chain ligand and is probably bound to a main-chain carbonyl group. The region of density above Chl a6 is due to the phytol chain. The density to the

A runs from Pro 170 to Ile 199 in 8 turns, over a length of 43 Å. Both helices start with a proline and are tilted by 32° relative to the membrane normal. The peptide chain in the second trans-membrane helix (helix C; Fig. 2c) was traced in a similar way, using as guide points the bulky side-chain densities of Ile 124, Leu 125, Ile 127 and Trp 128 in the lower part, and Tyr 141,

Arg 142 and Ile 143 in the upper part of the helix. Helix C extends from Ser 123 to Ile 143 over a distance of 31 Å in 5.5 turns, with a tilt angle of 9° relative to the membrane normal. Helices A and B intertwine in a left-handed supercoil near the centre of the membrane. The region of close contact extends from Ser 69 to Ala 76 in helix B, and from Gly 184 to Met 191 in helix A, where the distance between the centres of the two helices is 8.5 Å (Fig. 2d).

The first 24 residues of helices A and B are related by an axis of local 2-fold symmetry running perpendicular to the membrane



right of helix C belongs to the adjacent monomer in the LHC-II trimer⁶. *d*, Cross-sectional view showing helices A, B and C at the level of closest contact between helices A and B. Small residues at the interface (Ser 69, Ala 72, Gly 75, Ala 76 in helix B; Gly 184 and Ala 187 in helix A) are flanked by larger side chains (His 68 and Met 73 on helix B, Asn 183 and Met 188 on helix A). Met 73 interacts with Gly 184 and Ala 187, and Met 188 with Ala 72 in a tongue-and-groove fashion. The symmetry-related tetrapyrrole rings of Chl a2 and a5 are liganded by His 68 and Asn 183. Parts of the tetrapyrrole rings of the symmetrical Chl pairs a1, a4 and b2, b5, and of the polyene chains of luteins 1 and 2 are also visible. *e*, Helix D (Pro 205 to Ala 214). The helix is amphipathic, with hydrophobic residues (Leu 209 and Leu 213) pointing upwards into the interior of the complex and acidic residues (Glu 207 and Asp 211) extending into the thylakoid lumen. The side-chain densities of Pro 205 and Phe 194 with a distance of 4.9 Å between ring centres are fully resolved at a higher contour level (Fig. 1*b*). The lower ends of luteins 1 and 2 are within hydrogen-bonding distance of side chains in the BC loop (left) and helix D. *f*, Lutein 1, sitting in the groove between the crossed helices A and B. The upper ring is within van der Waals distance of Chl a1. The lower end is similarly close to Chl a3. *g*, Lutein 2, related by the internal dyad to lutein 1 in the groove on the opposite side of the crossed helices A and B. Chl a4 and a6 in van der Waals contact with lutein 2 are related by the same symmetry to Chl a1 and a3. Stereospecific binding of both luteins is ensured by the presence of Met 73 and Met 188 in the grooves of the left-handed supercoil which would collide with an inward-facing lutein methyl group at this location. With the exception of *d*, the horizontal lines of the map grid are parallel to the membrane plane (1 vertical grid unit, 0.89 Å), with the stromal surface on top and the luminal surface below. In *d*, 1 grid unit is 0.925 Å. The 3D map was calculated using standard crystallographic programs (CCP4)⁵⁰, with experimentally determined phases and observed structure factors F_o only. Map contours were drawn at 1.85 times the standard deviation. The atomic model was built using the program O⁵¹. Lutein co-ordinates were adapted from β -carotene⁵². The precise orientation of the luteins and of most Chl tetrapyrroles is not known.

plane. The existence of this symmetry had already been suggested by the 6 Å map⁶ and by internal sequence homology^{7,16}. At 3.4 Å, it served as an independent control of the polypeptide fit. The 2-fold symmetry manifests itself in the position of pairs of bulky residues, including Phe 58 and Phe 173, Trp 71 and Leu 186, Met 73 and Met 188 and, most strikingly, in the arrangement of the charged residues Arg 70 and Arg 185, Glu 65 and Glu 180, which apparently form two ion pairs in the hydrophobic interior of the complex (Fig. 2*a, b*). The arginine side chains extend from a position near the centre of the membrane

TABLE 1 Electron crystallographic data	
Two-dimensional crystals	
Layer group	<i>p</i> 321
Unit cell	<i>a</i> = <i>b</i> = 129.5 Å
Thickness	48 Å
Electron diffraction	
Number of diffraction patterns merged	83
Resolution	3.2 Å
R_{sym} *	21.8%
R_{merge} †	27.6%
Sampled amplitudes to 3.2 Å	17,920
Maximum tilt	58.7°
Fourier space sampled	85.4%
Phase determination by image processing	
Processed images	79
Resolution	3.4 Å
Average phase residual	27.7°
Sampled phases to 3.4 Å	15,124
Maximum tilt	60.9°
Fourier space sampled	87.4%
Crystallographic refinement	
Crystallographic <i>R</i> factor (20 Å to 3.4 Å)‡	33.0%
Free <i>R</i> factor (20 Å to 3.4 Å)	37.9%

Conventional constrained refinement of the initial hand-built model was done with the programme X-PLOR¹⁵ without simulated annealing on an α -Vax computer, using electron scattering amplitudes^{17,44} with phase and amplitude constraints.

$$* R_{\text{sym}} = \sum_{h,k,z} |I_{h,k,z} - I_{-h,-k,-z}| / \sum_{h,k,z} (I_{h,k,z} + I_{-h,-k,-z})$$

$$† R_{\text{merge}} = \sum_{h,k,z} |I_{h,k,z}^{\text{obs}} - I_{h,k,z}^{\text{merged}}| / \sum_{h,k,z} I_{h,k,z}^{\text{merged}}$$

‡ The crystallographic *R* factor was calculated as defined by $R = \sum_{h,k,z} |F_{h,k,z}^{\text{obs}} - F_{h,k,z}^{\text{calc}}| / \sum_{h,k,z} F_{h,k,z}^{\text{obs}}$. The free *R* factor was calculated according to ref. 45.

towards the N-terminal surface so that the positively charged guanidinium groups are placed into juxtaposition with negatively charged glutamic acid side chains from the opposite helix. The two buried ion pairs would provide a strong attractive force between the two helices and are likely to play a major part in stabilizing the protein in the membrane.

The two arginine side chains are particularly well defined, whereas the densities for the glutamic acid side chains are weak or negative beyond the β -carbons. This apparent discrepancy is accounted for at least in part by the interaction of electrons with

charged residues. Electron scattering factors¹⁷ of the negatively charged carbonyl oxygens are strongly negative, so the side-chain density would appear weaker. Conversely, the positively charged guanidinium groups have positive electron scattering factors and would therefore be expected to show up more strongly, as observed. In addition, side chains containing comparatively fewer hydrogen atoms are expected to be less visible than aliphatic or aromatic residues because hydrogens contribute relatively strongly to electron scattering¹⁷. This is consistent

with our observation that many side-chain densities of acidic and amide residues are weak. Other possible explanations, such as noise or side-chain flexibility, cannot be ruled out but seem less likely.

Membrane surface

Of the 232 amino-acid residues of the polypeptide, 36% form transmembrane helices. The remainder are exposed on the inner (luminal) and outer (stromal) membrane surface. As expected,

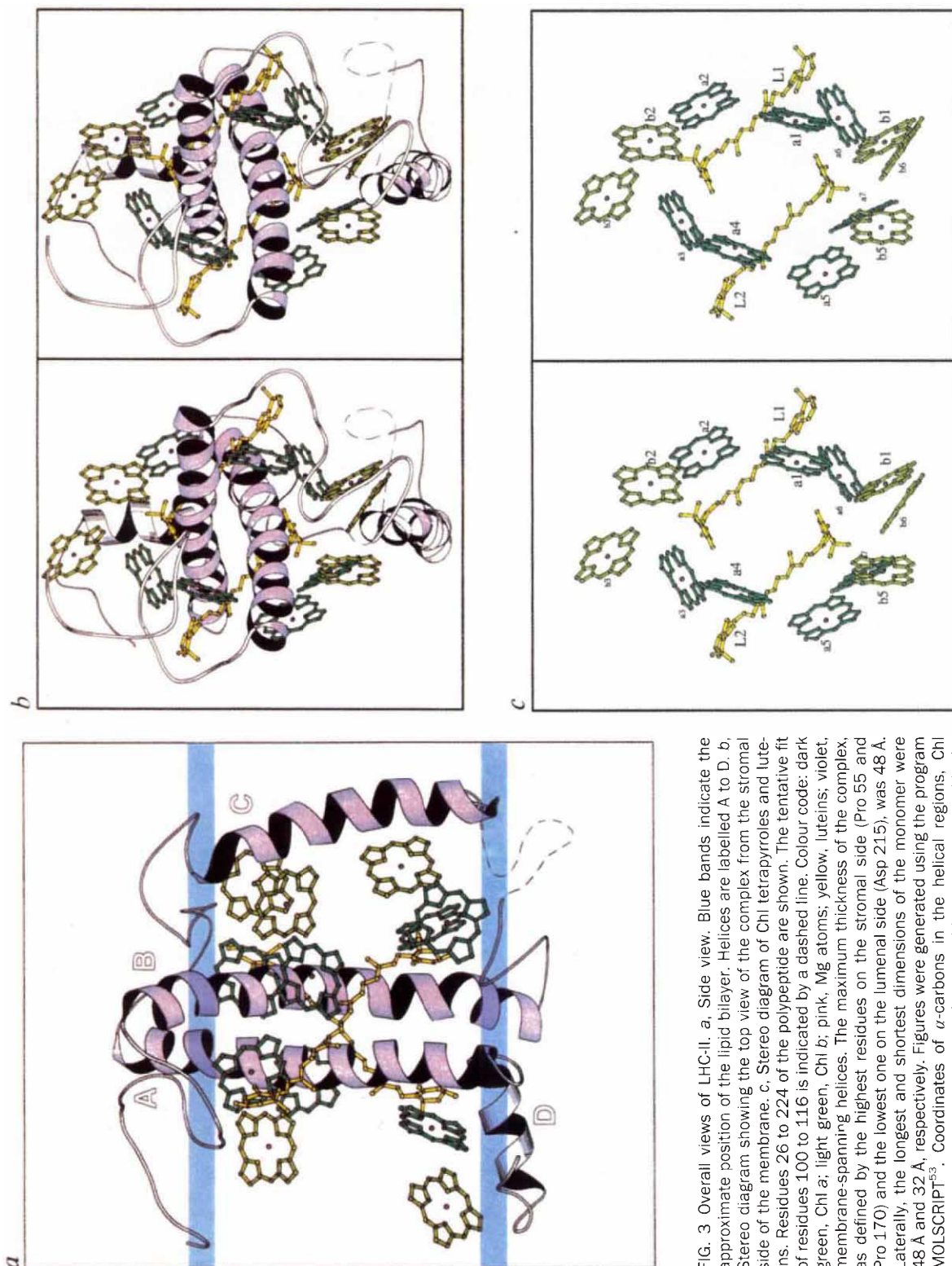


FIG. 3 Overall views of LHC-II. a, Side view. Blue bands indicate the approximate position of the lipid bilayer. Helices are labelled A to D. b, Stereo diagram showing the top view of the complex from the stromal side of the membrane. c, Stereo diagram of Chl tetrapyrroles and luteins. Residues 26 to 224 of the polypeptide are shown. The tentative fit of residues 100 to 116 is indicated by a dashed line. Colour code: dark green, Chl a; light green, Chl b; pink, Mg atoms; yellow, luteins; violet, membrane-spanning helices. The maximum thickness of the complex, as defined by the highest residues on the stromal side (Pro 55 and Pro 170) and the lowest one on the luminal side (Asp 215), was 48 Å. Laterally, the longest and shortest dimensions of the monomer were 48 Å and 32 Å, respectively. Figures were generated using the program MOLSCRIPT³³. Coordinates of α -carbons in the helical regions, Chl tetrapyrroles and polyene chains of carotenoids will be deposited in the Brookhaven data bank and are available on request.

features in the loop regions are less clearly defined than the transmembrane helices. However, stretches of 9 residues each at the N-terminal ends of helices A and B (Trp 46 to Asp 54 and Phe 161 to Asp 169) are related by the same local 2-fold symmetry, which again provided an internal control of the polypeptide fit. Hydrophobic residues, notably Trp 46 and Phe 161, Leu 51 and Leu 166, are accommodated in densities of appropriate shape and size oriented towards the hydrophobic interior of the complex, whereas charged side chains are pointing upwards into the solvent. The loop between helices C and A was fitted completely. Towards the N terminus, the peptide chain was traced to Gly 26. Beyond this, the features of the map are less distinct. The first eight amino-acid residues of the polypeptide are probably disordered¹⁸. They are exposed on the stromal surface of the membrane¹⁹ and contain the site of LHC-II phosphorylation^{2,20}. The first 54 residues of the polypeptide and the loop connecting helices C and A (Ala 144 to Asp 169) are therefore located on the stromal side, whereas the loop joining helices B and C (Val 90 to Gln 122) and the C-terminal portion of the polypeptide (Val 200 to Lys 232) are on the luminal side.

A fourth α -helix of almost three turns, referred to as helix D (Fig. 2e), was discovered at the interface between the hydrophobic interior and the thylakoid lumen (residues Pro 205 to Ala 214). Like the long transmembrane helices A and B, it starts with a proline. It has pronounced amphipathic character, with hydrophobic residues Leu 206 and Leu 209 extending into the hydrophobic interior, and acidic residues (Glu 207 and Asp 211) pointing into the thylakoid lumen. The density for the 8 C-terminal residues (Ala 225 to Lys 232) is weak. This part of the model is therefore tentative. In the BC loop, the polypeptide was traced to Ala 100 and again from Pro 116. For the intervening 15 residues an unambiguous fit is not possible at present.

Structural role of carotenoids

The major xanthophyll component in LHC-II is lutein²¹, of which two molecules have been found per monomer^{22,23}. Two 30-Å-long regions of density in the centre of the complex, which have the shape and size of carotenoids, were assigned to lutein (Fig. 2f, g). These are situated on either side of the helices A and B, sitting in the grooves of the supercoil, and are related by the local dyad (Fig. 3). The polyene chains of both molecules include an angle of $\sim 50^\circ$ with the membrane normal. The closest distance between them, at a point near the centre of the membrane, is ~ 11 Å. The polypeptide environment of the two polyene chains is different (Fig. 2f, g), but in both cases is highly hydrophobic. Their slightly curved S-shape suggests that they are in the all-*trans* conformation.

The head groups of the luteins are roughly equidistant from both membrane surfaces, in agreement with results obtained by resonance Raman spectroscopy²⁴, and are within hydrogen-bonding distance of the polypeptide loops on the membrane surfaces (Fig. 3a, b). Lutein 1 is probably attached to Gln 197 on the luminal side and to a residue between Ser 160 and Leu 164 on the stromal side. For lutein 2, the likely attachment sites are between Asp 47 and Ala 49 on the stromal side, and between Trp 97 and Ala 100 on the luminal side. The xanthophyll binding sites on the stromal side thus seem to be located in the highly conserved, symmetrical extension of helices A and B. The luteins form an internal cross-brace in the centre of the complex (Fig. 3a, b), providing a direct, strong link between the peptide loops at both surfaces. This may be necessary because of the comparatively open structure of the polypeptide (Fig. 3a), which is mainly involved in pigment binding so that tight helix-helix interactions are confined to only one site. By comparison, such interactions are numerous in bacteriorhodopsin¹² and the bacterial reaction centre⁴. The central position of the xanthophylls in LHC-II explains why they are essential for the *in vitro* reconstitution of the complex^{25,26}. The other carotenoids associated with LHC-II, primarily neoxanthin, participate in the xanthophyll cycle²⁷ and are present in sub-stoichiometric

amounts²². They are therefore likely to be located at the periphery of the complex.

Photoprotection

Photochemically, carotenoids have a dual role in photosynthetic antenna complexes: a light-harvesting function, augmenting energy absorption by chlorophyll, and a protective function. The latter is more important in green plants as the carotenoids do not make a major contribution to the absorption spectrum of LHC-II (ref. 21). The photoprotective function of carotenoids is due to their unique ability to desensitize the chlorophyll triplet state, which would otherwise result in the formation of highly reactive singlet oxygen^{28,29}, which is lethal to all forms of life. O₂ is produced in immediate proximity to LHC-II by the water-splitting photosystem II reaction centre. It is a triplet in the ground state and as such reacts readily with triplet chlorophyll, which forms by spin inversion of weakly coupled electrons in the excited state. The risk of singlet oxygen formation and hence of photodamage is particularly severe because chlorophyll triplet states are long-lived (~ 1 ms; see ref. 30) as relaxation to the singlet ground state is spin-forbidden.

Carotenoids quench chlorophyll triplets very efficiently through an electron exchange mechanism³¹ which requires partial overlap of the wave functions of the participating molecules. The map shows that chlorin rings of seven chlorophylls are in van der Waals contact with a carotenoid and thus close enough for direct electron transfer (Fig. 3 and Table 2). The close association of chlorophylls and carotenoids is a prerequisite for oxygenic photosynthesis. The spatial relationship between carotenoids and chlorophylls in other photosynthetic pigment-protein complexes can therefore be assumed to be very similar.

Chlorophylls a and b

In intact LHC-II, energy absorbed by chlorophyll *b* (Chl *b*) is transferred to Chl *a* within less than 1 ps (refs 32, 33), where it remains for 1 to 3 ns, as shown by the Chl *a* fluorescence-decay time in the purified detergent-solubilized complex³⁴. Energy

TABLE 2 Chlorophyll ligands and distances

Chlorophyll ligands				
Chl a1	Glu 180	Chl b1	Not known	
Chl a2	Asn 183	Chl b2	Not known	
Chl a3	Gln 197	Chl b3	His 212	
Chl a4	Glu 65			
Chl a5	His 68	Chl b5	Glu 139	
Chl a6	Gly 78 C=O	Chl b6	Gln 131	
Chl a7	Not known			
Lutein-chlorophyll distances (Å)*				
Lutein 1	Chl a1	4.0	Chl b1	10.7
	Chl a2	3.7	Chl b2	8.9
	Chl a3	4.1	Chl b3	7.9
Lutein 2	Chl a4	4.2		
	Chl a5	4.0	Chl b5	8.0
	Chl a6	4.9	Chl b6	6.5
	Chl a7	4.9		
Chlorophyll a-chlorophyll b distances				
		Ring distance [†] (Å)	Mg-Mg distance (Å)	
Chl a1	Chl b1	5.0	10.5	
Chl a2	Chl b2	4.0	8.3	
Chl a3	Chl b3	4.0	9.2	
Chl a5	Chl b5	4.6	8.9	
Chl a6	Chl b6	4.1	8.8	
Chl a7	Chl b6	4.0	10.0	

Distances are accurate to within ~ 1 Å. In the 6 Å map of LHC-II (ref. 6), 15 regions of density had been attributed to chlorophylls. Of these, 12 were confirmed at higher resolution. Densities numbered 12 and 13 in the 6 Å map were found to be due to aromatic amino-acid side chains. Density 4 in the 6 Å map may belong to a chlorophyll but this would be unique in not making contact with any other pigment molecule in the same monomer.

* Closest distance between atoms of the lutein polyene chain and chlorophyll tetrapyrrole.

† Closest distance between atoms of tetrapyrrole rings.

transfer from Chl *b* to Chl *a* is thus very much more rapid than the formation of chlorophyll triplets, which takes several nanoseconds^{35,36}. It follows that triplet quenching is only required for Chl *a*. This makes it possible to assign the seven chlorophylls closest to the carotenoids to Chl *a* (Table 2 and Fig. 3). The five chlorophyll head groups adjacent to another chlorophyll but not to a carotenoid probably belong to Chl *b*. This assignment is preliminary and needs to be confirmed at higher resolution as the chemical difference between Chl *a* and Chl *b* (a formyl group instead of the methyl group at the 7-position in the chlorin ring of Chl *a*) is too small to be detected at 3.4 Å. Spectroscopic measurements of carotenoid triplets in LHC-II have shown, however, that chlorophylls *a* are indeed closer than chlorophylls *b* to the carotenoids³⁷. Preliminary calculations indicate that the time for energy transfer from the presumed chlorophylls *b* to the chlorophylls *a* closest to them is 0.2 ps or less, in excellent agreement with the experimentally determined Chl *a* to Chl *b* energy transfer rates^{32,33}.

Lutein 1 is in contact with three chlorophylls, referred to as *a*1, *a*2 and *a*3, at minimum atomic distances of 3.7–4.1 Å (Table 2). The distances between lutein 2 and Chl *a*4, *a*5 and *a*6 are similarly short. Like the two carotenoids, the two sets of Chl *a* molecules are related by the internal dyad (Fig. 3c). The symmetry is unlikely to be exact, given that the protein environments on both sides are slightly different. Its functional significance is not known, but it may be important for excitonic coupling of chlorophylls and for Förster transfer to other antenna complexes and reaction centres⁶. The chlorin ring planes of the Chl *a* molecules are roughly parallel to the polyene chains of the luteins (Fig. 3). The Chl *a* referred to as *a*7 appears to be the only one without a symmetry mate. It is less close to lutein 2 (Table 2) than the others and may in fact be a Chl *b*. Chlorophylls *a*1, *a*2, *a*3, *a*5 and *a*6 are each in touch with a Chl *b*, referred to by the same number (Table 2). Chl *a*4 is not in van der Waals contact with another chlorophyll and may have a special function. Chl *a*7 is in contact with Chl *b*6. Together, chlorophylls *a*6, *a*7 and *b*6 form a closely spaced cluster near the luminal surface in a cavity lined by the lower ends of helices A, B and C. Only two Chl *b* molecules (*b*2 and *b*5) appear to be symmetry-related to one another (Fig. 3c).

The densities of the chlorin rings of all chlorophylls *a* and three chlorophylls *b* (*b*1, *b*5 and *b*6) are particularly well defined (Fig. 2). Those of Chl *b*2 and *b*3 are less clear, either because their chlorin rings are less steeply inclined with respect to the membrane plane and hence not as well resolved, or because they are less ordered owing to their more peripheral position. A bulky region of density near the N terminus of the polypeptide may belong to a chlorophyll, but this would be unique as it makes no contact with another, more centrally located pigment molecule. It is therefore more likely that this density belongs to another component of LHC-II, such as the part of the polypeptide, or bound lipid¹⁸. The 3.4 Å map thus shows that the complex contains 12 or, at most, 13 molecules of chlorophyll. Biochemical analysis³⁸ of crystalline LHC-II indicated a total of 14 chlorophylls per polypeptide and a Chl *a*/Chl *b* ratio of 1.3 (S. Nussberger and W.K., unpublished results), or eight Chl *a* and six Chl *b* per monomer. Although this ratio is roughly consistent with the proposed assignment of chlorophylls to Chl *a* and *b*, the experimentally determined chlorophyll content suggests that some chlorophyll remains nonspecifically bound to the complex throughout purification and crystallization.

Chlorophyll ligands

The chlorophylls are attached to the polypeptide by coordination of the central magnesium atom to polar amino-acid side chains or to main-chain carbonyls in the hydrophobic interior of the complex. So far, eight side-chain ligands have been identified (Fig. 4 and Table 2). Only two of these are histidines (His 68 and His 212), which are the sole amino-acid side-chain ligands in bacteriochlorophyll-protein complexes with known

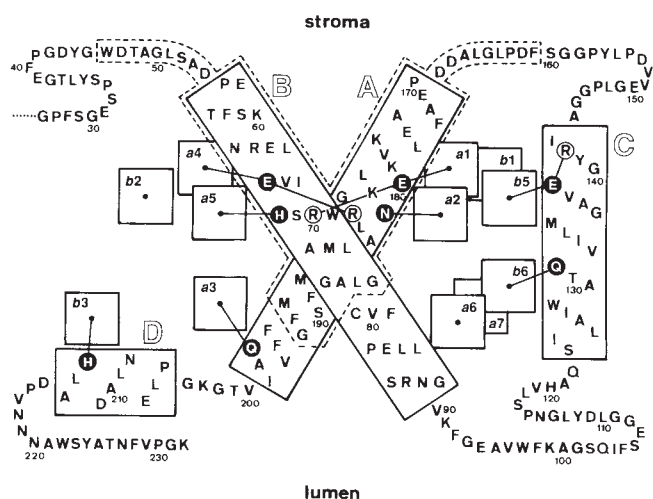


FIG. 4 Plan of the LHC-II polypeptide in the thylakoid membrane. Letters on black background indicate Chl side-chain ligands. Glutamate side chains that are presumed to act as Chl ligands are connected by a line to the charge-compensating arginines close to them, marked by open circles. The relative *z* positions of amino-acid residues in the membrane-spanning helices B, C, A (outlined capitals) and of the Mg atoms (black dots) in the centres of Chl tetrapyrroles (squares) are approximately correct. The dashed outline delineates regions of the polypeptide related by internal homology in the sequence and by local 2-fold symmetry in the structure.

structures^{4,39}. In LHC-II, three side-chain ligands are amides (Gln 131, Gln 197, Asn 183). Surprisingly, another three appear to be charge-compensated glutamates, forming ion pairs with arginines either in the same helix (Glu 139–Arg 142), or in another helix (Glu 65–Arg 185 and Glu 180–Arg 70). Glu 65 and Glu 180 thus seem to serve a dual function in chlorophyll binding and in locking together the two long transmembrane helices. The guanidinium groups of Arg 70 and Arg 185 are likely to be coplanar with the symmetrical chlorin rings of Chl *a*2 and *a*5, respectively, interacting through partial overlap of their π electron orbitals. Pairs of charged residues close to these two chlorophylls are bound to cause an electrochromic shift⁴⁰ of their absorption bands. Similar environmental effects are likely to influence the absorption characteristics of other chlorophylls in the complex. For example, the π electrons of Chl *b*5 seem to interact with the coplanar Trp 71 that intercalates between Chl *b*5 and *b*1.

Chl *a*6 appears to be liganded by the peptide carbonyl of Gly 78, which has no hydrogen-bonding partner owing to the location of Pro 82 in helix B. The distance between the centre of Chl *a*6 and the polypeptide chains seems too long (about 4.6 Å) for direct coordination, suggesting that a water molecule is involved. The remaining three chlorophylls (*b*1, *b*2 and *a*7) are likely to be bound in a similar way by peptide carbonyls in the loop regions. A polypeptide carbonyl and a water molecule are known to provide two of the seven magnesium ligands in the bacteriochlorophyll *a*-protein complex³⁹.

Evolutionary aspects

The polypeptide sequence of LHC-II is exceptionally highly conserved throughout the plant kingdom. This degree of structural conservation means that an optimal organization of pigment molecules has been achieved. All known Chl *b*-containing complexes in green plants^{41,42}, and even the Chl *a/c* complexes of chromophytic algae⁴³, share significant sequence homology with LHC-II and presumably have similar structures.

Helices A and B are invariant in all Chl *b*-containing antenna complexes⁴¹. The two helices, together with the six symmetrical

Chl *a* molecules and the two xanthophylls, seem to represent a central core of the complex. The internal 2-fold symmetry of this arrangement probably arose through duplication of a gene encoding an ancestral single-span polypeptide which proved effective in bringing Chl *a* into close contact with carotenoids. Helix C and the loops on the membrane surface show greater sequence variability within the family of Chl *a/b* binding

proteins⁴¹. It is striking that the Chl *b* molecules in the complex seem to be bound by these parts of the polypeptide, which may be later additions. This may account for the variable Chl *b* content of related antenna complexes⁴². The atomic model of LHC-II presented here will be refined in future studies at higher resolution, to contribute to a more complete understanding of photosynthesis which supports all life on Earth. □

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LETTERS TO NATURE

Detection of soft X-rays from supernova 1993J six days after outburst

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the detailed study of the evolution of a supernova in all wavelength regimes. We report here the detection of soft X-ray emissions from SN1993J, six days after the initial discovery, and the subsequent evolution of the X-ray light curve over the next 41 days. The spectral characteristics of the emissions can be readily explained if the X-rays originate in strong shock fronts produced by the rapid expansion of the supernova ejecta into the slow, dense wind of a red supergiant progenitor star⁴. The low intrinsic absorption of the earliest emissions requires that any circumstellar material is ionized, probably by the intense radiation of the initial outburst. The decay of the X-ray luminosity with time should provide important constraints on the density profiles of both the circumstellar gas and the outermost layers of the supernova ejecta.

The mission programme of the X-ray observatory Rosat was interrupted on 3 April 1993 for a target-of-opportunity observation of SN1993J. Using the position sensitive proportional counter (PSPC) in the focal plane of the X-ray telescope, two observations were made between 10.00 and 12.00 UT for a total of 2,706 s. Using former Einstein⁵ and Rosat observations of M81 a new source amidst the known bright X-ray sources in the field could be immediately identified with the supernova. The detection, a first estimate of the source intensity, spectral analysis results and the initial decrease of the X-ray intensity were reported⁶. Two days after the Rosat observation, Tanaka *et al.*⁷ reported the detection of X-rays from SN1993J with the Japanese X-ray observatory ASCA. Additional Rosat PSPC observations of the supernova followed throughout April until 6 May. In addition to the PSPC observations, two observations

ON 28 March 1993, a new supernova was discovered¹ in the nearby galaxy M81. The proximity of the event (the distance² to M81 is only 3.6 Mpc), and the fact that the supernova was detected at an early stage of its outburst³, makes SN1993J an ideal candidate for

with the high resolution imager (HRI) detector of the Rosat telescope were carried out. Table 1 summarizes the individual observations with both detectors. The angular proximity between M81 and the Sun prevented further monitoring of the source beyond mid-May.

Figure 1 shows contour plots of the central region of M81 before and after the supernova outburst. Figure 1a originates from a 20×10^3 s observation taken with the Rosat PSPC in September 1992 before the supernova explosion. In Fig. 1b, resulting from the combination of all PSPC pointings on SN1993J (27.3×10^3 s), the supernova is clearly visible as a new bright source southwest of the nucleus. By measuring the angular distance of SN1993J relative to the known positions⁵ of the nucleus of M81 and of the nearby source, we could verify that the X-ray and the optical⁸ positions coincide within the 6-arcsec accuracy of our approach. Because of the narrow point spread function of the PSPC, the data of SN1993J are only slightly contaminated (by at most 3%) by the nearby southeastern source, which is of similar brightness (and spectral shape) as the supernova. Thus a significant influence of that source on the analysis of SN1993J is excluded.

Figure 2 shows the evolution of the count rate of SN1993J over the whole observation period of 41 d. Two of the data points (indicated by circles) have been derived from the HRI data and scaled to the PSPC data according to the spectral parameters discussed below (with PSPC rate equal to 2.76 times the HRI rate). The horizontal bars of the data points indicate the time span over which data have been averaged to obtain comparable statistics on most points. An exponential decay fits the data in Fig. 2 slightly better than a linear decrease. The resulting ($1/e$) time is 86.2 d (+33.7, -19 d at 90% confidence).

For a spectral analysis of the data, each observation was initially treated separately. Power law and thermal fits were tested on each data set. No statistically significant change of the spectral parameters with time could be established for either of the two models. The data were then added to form a composite spectrum with better statistics.

The fit of a power-law model to the mean spectrum (Fig. 3) yields a photon index of 1.0 ± 0.25 ($\chi^2_{\text{red}} = 0.99$; 13 degrees of freedom, d.f.). The low energy cut-off of the spectrum is due to photoelectric absorption in foreground diffuse matter, and corresponds to a neutral hydrogen column density N_{H} of $(5.9 \pm 0.7) \times 10^{20} \text{ cm}^{-2}$. For a fit to a thermal bremsstrahlung model the restricted energy range of the PSPC (0.1–2.4 keV) does not allow kT (where k is the Boltzmann constant and T is

TABLE 1 Rosat observations of SN1993J

Detector	Days (in 1993)	Observation time (s)	rate counts s^{-1}
PSPC	93.5	2,705	0.0790 ± 0.0055
PSPC	98.9	4,060	0.0734 ± 0.0043
PSPC	102.6	5,479	0.0609 ± 0.0034
PSPC	106.8	514	0.0614 ± 0.0121
HRI	109.2	13,210	0.0211 ± 0.0014
PSPC	113.1	5,464	0.0554 ± 0.0032
PSPC	125.3	9,051	0.0535 ± 0.0025
HRI	134.2	8,044	0.0159 ± 0.0016

Exposure times and uncorrected count rates (in the 0.1–2.4 keV band) for Rosat pointings on SN1993J with the position sensitive proportional counter (PSPC) and the high resolution imager (HRI) detectors. Using the measured spectral parameters, the PSPC rate equals 2.76 times the HRI rate.

temperature) to be constrained at the high temperature side. We find $kT > 7 \text{ keV}$ (or $T \geq 80 \times 10^6 \text{ K}$) at a 1σ confidence level. The corresponding N_{H} column density is $(5.9 \pm 1.1) \times 10^{20} \text{ cm}^{-2}$ ($\chi^2_{\text{red}} = 1.05$; 13 d.f.). The galactic value of $4.3 \times 10^{20} \text{ cm}^{-2}$ in the direction towards M81, determined from radio measurements⁹, shows that most of the absorbing material along the line of sight is located within our galaxy. Thus our measurement excludes the existence of large amounts of X-ray absorbing material near the source.

Assuming a distance² of 3.6 Mpc to M81 and the best-fit value for the power law model, the observed flux in the 0.1–2.4 keV band corresponds to an absorption corrected luminosity of $(2.94 \pm 0.20) \times 10^{39} \text{ erg s}^{-1}$ on 3 April and of $(1.64 \pm 0.17) \times 10^{39} \text{ erg s}^{-1}$ in mid-May. Besides statistical uncertainties the luminosity has an additional error of $\sim 25\%$ due to the uncertainty of the spectral parameters.

Models for early X-ray emission from SN1993J have to be tested against the present findings of the very high temperatures in the emission region, the low intrinsic absorption, the fact that X-rays were detected 6 days after the explosion, as well as against the slow decay of the soft X-ray luminosity. A rather obvious explanation for the present observations is provided by models where the interaction between the shock wave from the supernova and the circumstellar material, originating from the dense wind of a red supergiant progenitor star, heats matter to very high temperatures⁴. The soft X-ray luminosity requires high densities of the circumstellar matter, typical for the dense winds

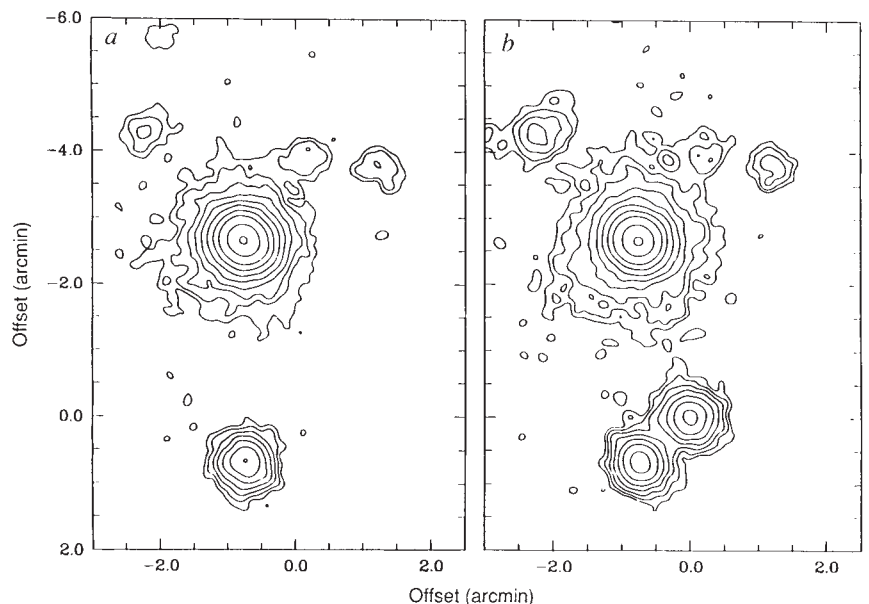
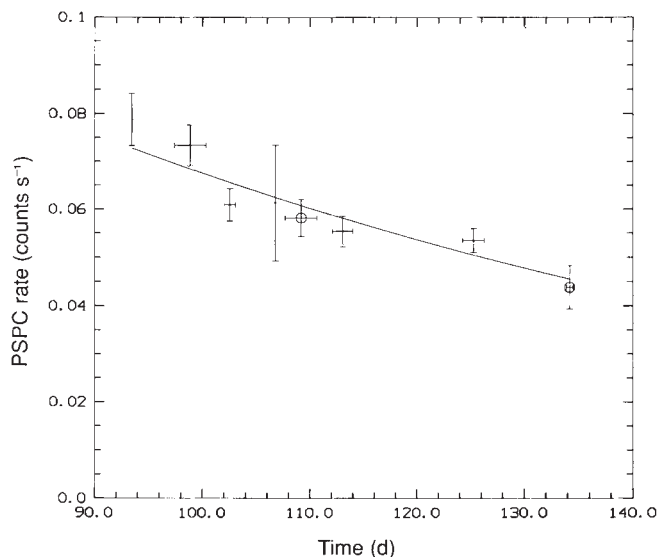


FIG. 1 Contour plots from weakly smoothed images of the central region of the galaxy M81. a, A 20×10^3 s exposure taken with the position sensitive proportional counter (PSPC) on the Rosat satellite in September 1992. b, The same region after the supernova explosion (27.3×10^3 s PSPC data). The distance between the M81 nucleus at the centre and the bright X-ray source at the lower rim is ~ 3 arcmin. The contours begin at 3 standard deviations above the background and go up in steps of a factor 2. The PSPC point spread function for a supernova-like spectrum has a half-energy radius of ~ 14 arcsec.

FIG. 2 Light curve of SN1993J as measured with the PSPC detector on Rosat in the 0.1–2.4 keV band. (The horizontal axis gives the days in 1993.) The two data points with circles represent the high resolution imager (HRI) rates that have been scaled by a factor of 2.76 (using the measured spectral parameters) to comparable PSPC rates. The line represents an exponential decay fit with a decay time of 86.2 d.



of red supergiants, as confirmed by detailed hydrodynamical calculations by Suzuki *et al.*¹⁰. The initial spectrum of SN1993J can be explained reasonably well by thermal bremsstrahlung emission from the supernova ejecta heated by the reverse shock to electron temperatures of the order of 3×10^8 K. But this model predicts a much faster decrease of the soft X-ray luminosity than observed, resulting in almost a factor of 2 difference at day 47 after the explosion. Tuning the circumstellar gas density profile and the density distribution in the outermost layers of the ejecta may help to overcome this discrepancy (K. Nomoto, personal communication).

Before SN1993J, the only supernova event from which low-energy X-rays had been seen was SN1980K in NGC6946, which was detected¹¹ at 5 standard deviations above background with the Einstein observatory 35 days after maximum optical light; 32 days later, the source was no longer visible. Canizares *et al.*¹¹ estimated a luminosity of 2×10^{39} erg s⁻¹ (0.2–4 keV) during the first observation, remarkably close to the luminosity we find for SN1993J.

In marked contrast to SN1980K and SN1993J, only hard X-rays¹² (from the radioactive decay of Ni⁵⁶) but no early soft X-rays¹³ (<2.5 keV) were seen from SN1987A, which exploded in the Large Magellanic Cloud, ~70 times as close as M81. The progenitor star of SN1987A was identified as a blue supergiant, an object class with typically thin and fast winds, which blow out a low-density bubble around the star. Here the circumstellar wind density is so low that the X-ray radiation from the shock interaction stayed below the detection limit (1.5×10^{36} erg s⁻¹ at a distance of 50 kpc) of the Aschenbach *et al.*¹³ observation.

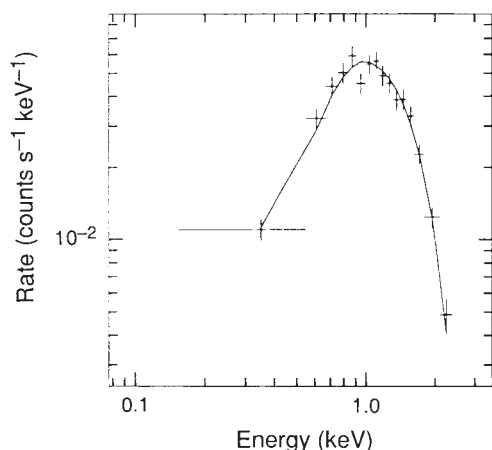


FIG. 3 Spectral fit of a power-law model of the form $f \propto E^{-\gamma}$ to the PSPC data of the supernova. Best fit values are $N_H = (5.9 \pm 0.7) \times 10^{20}$ cm⁻² for the (cold) gas absorption along the line of sight and $\gamma = 1.02 \pm 0.25$ for the photon index.

Returning to SN1993J we note that the low intrinsic absorption observed already on day 6 after the explosion requires that the wind material of the red supergiant progenitor has been efficiently ionized, for example by an initial ultraviolet or X-ray flash¹⁴. The hydrogen column density in units of 10^{22} cm⁻² outside the shock front along the line of sight, assuming a $1/r^2$ density distribution in the wind material, can be expressed as $N_{H,22} = 13 \times \dot{M}_{-5} / (v_{w,20} v_{s,4} t_d)^{-1}$. With the time after the explosion $t_d = 6$ in days, the wind velocity $v_{w,20} = 1$ in units of 20 km s⁻¹, the shock velocity $v_{s,4} = 3$ in units of 10^4 km s⁻¹ and mass loss rates $\dot{M}_{-5}$ of 0.6 to 7.7 in units of 10^{-5} solar masses per year (required to achieve the observed luminosity, see also Suzuki¹⁰), we get $N_{H,22}$ values of 0.5–6. If the wind matter was neutral, soft X-rays would be completely absorbed by the outer layers of the wind, in contradiction to the observation. In fact the fits to the Rosat spectra show a 90% confidence upper limit of 0.05 for $N_{H,22}$, which means that the wind is optically thin against photoelectric absorption for photon energies >0.3 keV at least. Furthermore, our observations are consistent with no change of the absorption column density until the end of the observation period with the PSPC detector, that is day 39 after the outburst. This indicates that the degree of ionization, effective in the soft X-ray range, was essentially maintained.

After submission of this paper, SN1993J was re-observed by Rosat on 1 and 2 November 1993¹⁵. Since the last observation in May 1993, the PSPC count rate between 0.1 and 2.4 keV has decreased from 0.053 ± 0.0025 to 0.035 ± 0.0014 counts per second. The energy spectrum has become significantly softer, with $kT \approx 0.5$ keV for the fit to a thermal bremsstrahlung model. The low-energy X-ray absorption, as derived from the fit, has increased by a factor of ~5 compared to the May observation. □

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The orbit and atmospheric trajectory of the Peekskill meteorite from video records

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ON 9 October 1992, a bright fireball appeared over West Virginia, travelled some 700 km in a northeasterly direction, and culminated in at least one impact: a 12.4-kg ordinary chondrite was recovered in Peekskill, New York¹. Fortunately, the event was captured on several video recordings, which provide a detailed record of both the fragmentation of the object and related atmospheric effects. These are the first motion pictures of a fireball from which a meteorite has been recovered. We report here the preliminary analysis of 14 video recordings of the event, from which we determine the ground path and the original orbit of the object.

Objects responsible for bright fireballs occupy the low-mass ($1\text{--}10^6$ kg) end of the asteroid size spectrum. Several recent studies^{2,3,4} have considered the fragmentation of large meteoroids during atmospheric flight, but comparison of these models with observational data has not been possible. Up to now, only three meteorites have been recovered^{5,6,7} for which detailed data exists on their atmospheric trajectory and orbit. Consequently, there are few constraints on the numerical models of meteoroid entry dynamics and the position of meteorites in the Solar System before impact on Earth.

At 23:48 UT (± 1 min) on 9 October 1992, a fireball, brighter than the full moon, appeared over West Virginia. The fireball travelled in an approximately northeasterly direction with an estimated luminous flight time in excess of 40 s, covering a ground-path of >700 km (Fig. 1). During the second half of its flight, the fireball exhibited extensive fragmentation with several dozen individual fragments visible on some video frames. These fragments reached a maximum simultaneous separation of >20 km, with at least 70 pieces visible on two higher-resolution still photographs of the event. Two main pieces were visible in the last part of the trajectory. A fragment struck a parked car, at Peekskill, New York ($41^\circ 17' \text{ N}$, $73^\circ 55' \text{ W}$). Many eye-witness accounts of the fireball were received and at least 14 video recordings of the event were made.

The results presented here are based on triangulation analyses using video data from four locations: Fairfax, Virginia ($38^\circ 51' \text{ N}$, $77^\circ 19' \text{ W}$); Johnstown, Pennsylvania ($40^\circ 20' \text{ N}$, $78^\circ 56' \text{ W}$); Pittsburgh, Pennsylvania ($40^\circ 26' \text{ N}$, $80^\circ 01' \text{ W}$); and Willoughby, Ohio ($41^\circ 38' \text{ N}$, $81^\circ 26' \text{ W}$). The location of these stations is indicated in Fig. 1. The most comprehensive view of the event is provided by the Johnstown video, which records ~ 22 s of the flight. From each station the individual video frames were digitized and measured. This analysis is based on measurement of 254 points. The multi-station analysis procedures are fully described elsewhere^{8,9}.

The apparent radiant (the point on the celestial sphere from which the meteor appears to originate) is $\alpha = 15 \text{ h } 07 \text{ min } \pm 02 \text{ min}$, $\delta = -16.2^\circ \pm 0.2^\circ$ (epoch 2000.0). The trail is extremely shallow. At the start of the video records (a height of 46.4 km), the trail makes an angle of only 3.4° with the horizontal. The last point measured on the video records

TABLE 1 Orbital parameters for the Peekskill meteorite

a (semimajor axis)	$1.49 \pm 0.03 \text{ AU}$
e (eccentricity)	0.41 ± 0.01
q (perihelion distance)	$0.886 \pm 0.004 \text{ AU}$
ω (argument of perihelion)	$308^\circ \pm 1^\circ$
Ω (longitude of ascending node)	$17.030^\circ \pm 0.001^\circ$
i (inclination)	$4.9^\circ \pm 0.2^\circ$
T (orbital period)	$1.82 \pm 0.05 \text{ yr}$
DT (time since perihelion)	$41 \pm 1 \text{ d}$
Q (aphelion distance)	$2.10 \pm 0.05 \text{ AU}$

corresponds to a height of 33.6 km, although this is not the end of the luminous path. This Earth-grazing fireball would have returned to space were it not for the Earth's atmosphere. Indeed, if it were ~ 40 km higher at perigee it would have skipped out of the atmosphere as did the 10 August 1972 Wyoming fireball¹⁰.

These video recordings present a wealth of time-resolved dynamical detail never before recorded for a fireball-meteorite event. In Fig. 2 we show a composite of the fireball as recorded from Johnstown. In the first part of the recorded luminous trajectory there is significant wake behind the fireball, but no distinct fragments. By a height of ~ 41.5 km, fragmentation into individual pieces is clearly visible. Differential aerodynamically induced lag makes it possible to discern several dozen fragments of different sizes by the time the object has reached a height of 38.6 km. The detail of this fragmentation is more clearly visible in the higher-resolution still photograph (Fig. 3). As well as a maximum longitudinal fragment displacement of >20 km, there is a much smaller but significant transverse displacement. There is a significant flare at a height of 36.2 km, followed by a burst of material which rapidly decelerates relative to the main fragments. In the last part of the trajectory there are four main fragments, with only the largest two being visible eventually. This would seem to indicate that there should be at least two,

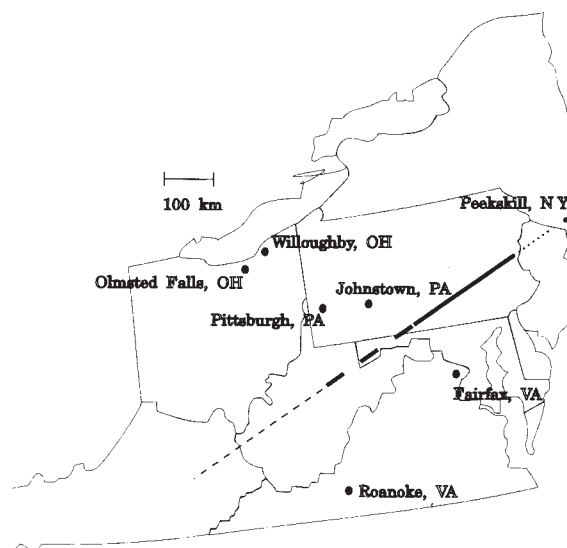


FIG. 1 Map of the northeastern United States showing the location of the stations used in the video analysis, the projection of the trajectory on the Earth's surface and the Peekskill meteorite recovery site. The ground track as actually recorded on video is the solid line; the dotted line represents the undocumented trajectory past the last recorded point; the thick dashed line is based on a compilation of visual observations for the early part of the trajectory; and the thin dashed line is a theoretical initial portion of the trajectory based upon the assumption of an 80-km height at the start of the luminous trajectory. NY, New York; OH, Ohio; PA, Pennsylvania; VA, Virginia.

FIG. 2 Set of images showing the changing appearance of the fireball as viewed from Johnstown (see Fig. 1). The relative time (in seconds) is given on each image (the absolute value of the time marker has no significance). Early in the flight the fireball had a central luminous core and a trailing wake, probably due to spraying of liquid droplets or very fine grains (the scale in the first 6 images is $\sim 5.7^\circ$ horizontal width). The length of the combined luminous trajectory is 3.7 km in image 24.78 s (height 46.3 km) and grows to 13.8 km in image 32.12 s (height 40.5 km). As illustrated in the two central images, the distribution of fragments changed dramatically over rather short time intervals. In the latter part of the trajectory (bottom images) the fragments became more clearly distinct, until only two main pieces are visible (on either side of the double stadium pole in the image) at relative time 43.73 (height 34.1 km) (original video recording by J. Derr).

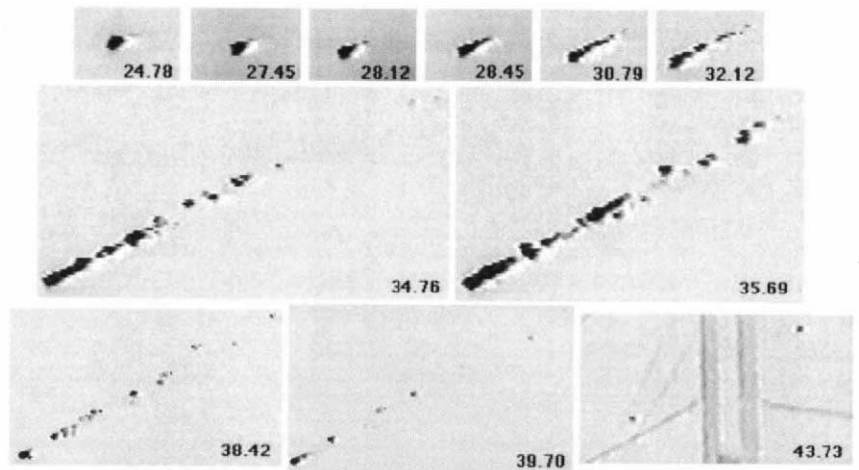
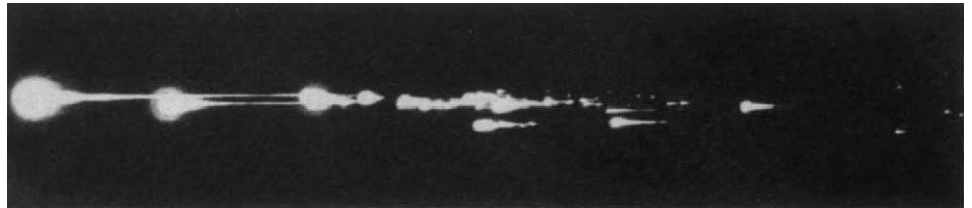


FIG. 3 Enlargement of a portion of a still photograph (by S. Eichmiller) taken from Altoona, Pennsylvania, showing extensive fragmentation. The photograph was shot on Kodak TMZ (3200) film with a 300-mm lens (shutter speed either 1/250 or 1/500 s, at an aperture of $f/4$ or $f/2.8$). The portion of the photograph reproduced here represents an angle of 3.4° . A minimum of 70 fragments are visible on the original photograph.



and possibly four or more, meteorite fragments on the ground, although only one has yet been found. The shallow trajectory makes the possible search area very large.

A number of observers reported that the fireball was significantly brighter than the full moon (which was visible in the sky at the time of the event, 97% illuminated at magnitude -12.8). The fireball saturated the dynamic response of the camcorders, and precise photometry is impossible. Work is in progress on approximate photometric measures, which will yield a photometric mass. Almost all observers referred to the strong green

hue of the fireball although this colour does not appear strongly in the video recordings owing to the spectral response of the CCD (charge-coupled device) photodetectors.

The video record shows the distinct presence of a flickering phenomena in the fireball's pre-fragmentary (above 43 km) flight. Frame-by-frame analysis of several video segments indicates that the plasma tail, or wake, periodically disconnects from the main body of the fireball. An average disconnection frequency of 6 Hz is found. Such a phenomenon has been reported previously¹¹, although at higher disconnection frequencies. We suggest that the disconnection events may be driven by the rotation or aerodynamic oscillation of the pre-fragmented parent body. These mechanisms associate the disconnection events with a modulated ablation rate. The disconnection events may alternatively be associated with a hydrodynamic instability in the fluid layer of the pre-fragmented meteoroid. This mechanism, on small scales, has recently been discussed¹².

As all the images were recorded in response to the spectacular event, the initial part of the trajectory was not obtained. However, the data suggest that the meteoroid had not suffered significant deceleration before the first video recording. We assumed a frame rate of 30 frames per second (60 video fields per second). The crystal-oscillator-based timing on the camcorders used should result in an error of no more than 0.1%. The analysis yields a pre-atmospheric velocity of $14.72 \pm 0.05 \text{ km s}^{-1}$, whereas the velocity for the last measured intervals was $\sim 5 \text{ km s}^{-1}$. The spatial accuracy of individual points is inferior to photographic meteor work, but the duration of the event (10–20 times longer than typical fireballs), the use of data from four widely spaced stations, the large number of points used (254), and the excellent (1/60 s) time resolution all contributed to a rather precise pre-atmospheric velocity and apparent radiant. Nevertheless, considering that the raw data were collected by mobile non-scientific instruments, and that a celestial reference object was available only at one station, perhaps the probable error should be

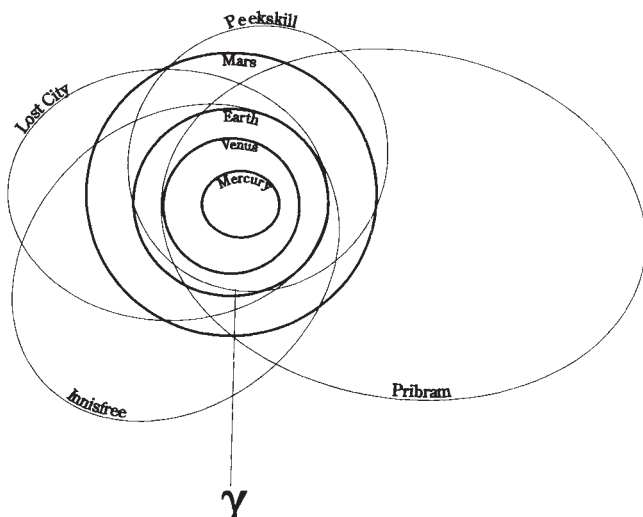


FIG. 4 The orbit of the Peekskill meteorite, along with the three previous meteorites with photographically determined orbits (Lost City, Pribram and Innisfree). The symbol γ is the Vernal Equinox.

increased by a factor of two or more to allow for possible uncorrected systematic errors.

Following corrections to the apparent radiant and velocity, to account for the gravitational attraction of the Earth and for rotation of the Earth, one obtains a corrected radiant of $\alpha = 13\text{ h } 56\text{ min } \pm 02\text{ min}$, $\delta = -29.3^\circ \pm 0.2^\circ$ (epoch 2000.0) with a geocentric velocity of $10.1 \pm 0.1\text{ km s}^{-1}$. It should be noted that the shallow trajectory required sophisticated analysis procedures¹⁰. The orbital parameters are given in Table 1, and the orbit is plotted in Fig. 4 along with the previous three meteorite orbits. In all cases the probable errors quoted are ± 1 standard deviation, subsequently multiplied by 2 to allow for possible uncorrected systematic errors. The object was encountered near perihelion, as are most meteorite-producing fireballs¹³.

Work is continuing on the further refinement of the atmospheric trajectory and the orbit. We anticipate improvements through measurements from additional digitized video frames, more reliable positional measurements of reference objects, possible incorporation of data from additional stations, and better modelling of deceleration. Other researchers are currently measuring the cosmogenic radioisotopes of the one recovered fragment (an H6 monomict breccia), which will provide indepen-

dent evidence relevant to the pre-atmospheric mass. In addition the rich video record should permit a detailed analysis of the fragmentation dynamics and flare phenomena. $\square$

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Path detection and the uncertainty principle

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QUANTUM mechanics predicts that any detector capable of determining the path taken by a particle through a double slit will destroy the interference. This follows from the principle of complementarity formulated by Niels Bohr: simultaneous observation of wave and particle behaviour is prohibited. But such a description makes no reference to the physical mechanism by which the interference is lost. In the best studied *welcher Weg* ('which path') detection schemes^{1,2}, interference is lost by the transfer of momentum to the particle whose path is being determined, the extent of momentum transfer satisfying the position–momentum uncertainty relation. This has prompted the question as to whether complementarity is always enforced in *welcher Weg* schemes by momentum transfer. Scully *et al.*³ have recently responded in the negative, suggesting that complementarity must be accepted as an independent component of quantum mechanics, rather than as simply a consequence of the uncertainty principle. But we show here that, in any path detection scheme involving a fixed double slit, the amount of momentum transferred to the particle by a perfectly efficient detector (one capable of resolving the path unambiguously) is related to the slit separation in accordance with the uncertainty principle. If less momentum than this is transferred, interference is not completely destroyed and the path detector cannot be perfectly efficient.

The best known *welcher Weg* detectors in a double slit arrangement include Einstein's recoiling slit¹ and Feynman's light-scattering arrangement². In these schemes and others⁴ it can be shown that the amount of momentum transferred satisfies the requirements of Heisenberg's uncertainty principle.

We now consider an arbitrary *welcher Weg* detector that determines the path taken by a particle through a fixed double slit. If the particle's position immediately after its passage through the double slit is x , then the detector state is $|D_x\rangle$. As the particle can only be located near the slit positions x_1 and x_2 , the detector

records the path of the particle by being left in state $|D_{x_1}\rangle$ or state $|D_{x_2}\rangle$. The joint state of particle and detector is described by the entangled (or correlated) state

$$|\Psi\rangle = \int dx \psi_i(x) |x\rangle \otimes |D_x\rangle$$

The wavefunction $\psi_i(x)$ is prepared by the double slit and consists of two narrow peaks at the slit positions x_1 and x_2 .

The path is determined after the interaction by making a measurement on the detector. The detector state may be written as a linear superposition of basis states $|\xi\rangle$,

$$|D_x\rangle = \sum_{\xi} O_{\xi}(x) |\xi\rangle$$

where the choice of basis depends on the type of measurement. The probability of obtaining the result ξ when the detector is in state $|D_x\rangle$ is $|O_{\xi}(x)|^2$. Given that this result has been obtained, the final wavefunction of the particle is

$$\psi_f(x) = N \psi_i(x) O_{\xi}(x)$$

where N is a normalization constant. Comparing the initial and final atomic wavefunctions $\psi_i(x)$ and $\psi_f(x)$, we see that the process of *welcher Weg* detection has multiplied the original wavefunction by a new distribution $O_{\xi}(x)$. This multiplication corresponds in momentum space (that is, after a Fourier transform) to convolving the initial atomic momentum distribution $\tilde{\psi}_i(p)$ with a 'momentum kick amplitude distribution' $\tilde{O}_{\xi}(p)$, which is the Fourier transform of $O_{\xi}(x)$,

$$\tilde{O}_{\xi}(p) = \frac{1}{\sqrt{2\pi\hbar}} \int dx O_{\xi}(x) e^{-ipx/\hbar}$$

$\tilde{O}_{\xi}(p)$ can be interpreted very naturally as the amplitude for transferring momentum p from the detector (namely the cavity system) to the atom, as the convolution process takes every momentum component p_i of the initial distribution $\tilde{\psi}_i(p_i)$, and produces new momentum components $\tilde{O}_{\xi}(p - p_i)$ in the final distribution $\tilde{\psi}_f(p)$.

We now suppose that for every outcome of the detector measurement the momentum transfer is limited. That is, for every ξ the momentum kick amplitude distribution $\tilde{O}_{\xi}(p)$ is zero outside the range $|p| < p_m$, where p_m is the maximum transferred momentum. In the terminology of Fourier transform theory,

the functions $O_\xi(x)$ are 'band limited'. We shall show that this restriction defines a lower bound for the visibility of the total interference pattern, and thus limits the localization efficiency.

The total interference pattern is the far-field distribution obtained by summing over all possible states of the detector after the measurement. Its visibility is equal to $\mathcal{V} = |\langle D_{x_1} | D_{x_2} \rangle|$, and may be regarded as a measure of the efficiency of localization, with unity indicating no localization, and zero signifying perfect localization efficiency. This interpretation can be understood as follows. If $\mathcal{V} = 1$, the detector states D_{x_1} and D_{x_2} are equal (up to some phase factor) and there is no way to distinguish the path of the particle. If $0 < \mathcal{V} < 1$, the detector will provide an inefficient position measurement because, regardless of the choice of detector basis, there will always be at least one possible outcome ξ for which both $\langle \xi | D_{x_1} \rangle$ and $\langle \xi | D_{x_2} \rangle$ are nonzero, leaving the path ambiguous. If the visibility is zero, then there will be at least one detector basis for which every possible outcome of the measurement process determines the path of the particle unambiguously.

We now define the function $v(x) = \langle D_{x_1} | D_x \rangle$, which we shall use to derive a lower bound for the visibility $\mathcal{V} = |v(x_2)|$. Noting that $v(x)$ is a linear combination of the functions $O_\xi(x)$, and is therefore band limited to the same band, we can establish the following properties of $v(x)$:

$$\begin{aligned} v(x_1) &= \langle D_{x_1} | D_{x_1} \rangle = 1 \\ |v(x)| &= |\langle D_{x_1} | D_x \rangle| \leq 1 \text{ for all } x \\ \tilde{v}(p) &= 0 \text{ for } |p| \geq p_m \end{aligned}$$

Using these conditions we can apply Bernstein's theorem⁵, which gives an upper bound on the derivative of a band limited function, to show that for all x ,

$$|v'(x)| \leq \frac{p_m}{\hbar}$$

The mean value theorem now gives us the desired lower bound on the visibility as a function of the maximum momentum transfer p_m and the slit separation d ,

$$1 \geq \mathcal{V} \geq 1 - \frac{p_m d}{\hbar}$$

Thus for a perfectly efficient *welcher Weg* detector (for which $\mathcal{V} = 0$), the maximum transferred momentum cannot be less

than that required by the uncertainty principle for the given slit separation:

$$p_m d \geq \hbar$$

We will now consider the ingenious *welcher Weg* scheme suggested by Scully, Englert and Walther³. Their detector consists of two empty micromaser cavities, placed one at each slit of a double-slit arrangement. The interaction time is chosen so that an excited atom passing through the double slit will emit a photon into one or other cavity. The cavities may be examined afterwards to determine which slit the atom went through. Scully *et al.* claim that this scheme does not involve any transfer of momentum between detector and particle; the fact that the interference fringes are nevertheless destroyed by the measurement suggests that the principle of complementarity must be accepted as an independent component of quantum mechanics, not a mere consequence of the uncertainty principle. But it has been argued^{6,7} that this scheme is in principle no different from the Einstein recoiling slit: in each case there is a random phase correlated with a variable that is conjugate by an uncertainty relation to the one measured. We now show that the physical origin of this randomization is momentum transfer.

There will always be some uncertainty in the transverse momentum of the emitted photon even if the cavity mode structure is chosen so that the photon momentum is primarily longitudinal. This is due to the fact that the photon is localized within the width w of the cavity. Using the uncertainty principle, we find that the minimum momentum uncertainty is of the order of $\hbar/2w$ where $\hbar$ is Planck's constant. For a slit separation d , the momentum separation between the maxima and minima of the double-slit interference pattern is $\hbar/2d$, so a single photon emission can account for the loss of interference if the slits are located at the centres of their respective cavities. If the width of the cavities is greater than the slit separation, a single photon does not carry sufficient momentum to destroy the interference pattern. In this case an explicit calculation⁸ gives the momentum kick distribution shown in Fig. 1. This distribution is the square modulus of the momentum kick amplitude distribution as defined in the general analysis given earlier. It is calculated for rectangular-box shaped cavities, and is independent of which cavity the photon was emitted into. Note that the momentum kick distribution extends beyond the range $|p| < \hbar/d$, as predicted by the general analysis. The distribution is smooth because it was calculated for cavities of width $\lambda/2$, where λ is the optical wavelength, which are so narrow that the photon momentum has a broad continuum of values. But the underlying structure of the distribution becomes evident if the cavities are extended outwards, keeping the wavelength constant, so that the transverse photon momentum becomes better defined. In the limit of very wide cavities the transverse photon momentum adopts the discrete values $\pm \hbar/\lambda$. The spikes in this comb distribution occur at odd multiples of the photon momentum, indicating that virtual processes occur during the interaction in which the photon is emitted and then reabsorbed from the opposite direction, thereby exchanging twice the photon momentum between the atom and the cavity walls. During the interaction time, many such virtual processes can occur before the final emission leaves the atom in its ground state.

An alternative analysis of the micromaser path detector, equivalent to the transverse momentum kick description, may be carried out in terms of longitudinal displacements of the atomic wavefunction. The sign of the interaction energy between the atom and the cavity depends on the atom's polarization with respect to the cavity field. If the energy is negative, the cavity mode acts as a potential well, and the atom's longitudinal velocity during the interaction will be higher than in free space. The atom leaves the cavity at the same longitudinal speed with which it entered (neglecting small corrections of the order of $\Delta v_\perp^2/v_\parallel$), but displaced forwards from the position it would have had in the absence of the cavity. If on the other hand the inter-

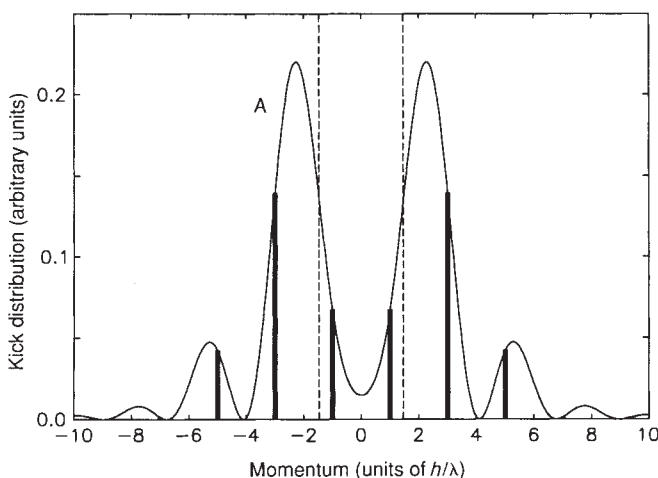


FIG. 1 The momentum kick distribution (curve A) for cavities of width $\lambda/2$. The underlying comb structure (thick black vertical lines) is the momentum kick distribution in the limit of very wide cavities. The chosen slit separation $d = (\lambda/\pi) \arcsin(1/3)$ is less than the width of the cavities. The vertical dashed lines delimit the range $|p| < \hbar/d$.

action energy is positive, the atom will travel slowly over the potential barrier formed by the cavity mode, and will be displaced backwards. Englert, Schwinger and Scully⁹ have shown that the atom will be displaced forwards or backwards by $\lambda_{dB}/4$, where λ_{dB} is the de Broglie wavelength of the atom.

The important quantity in determining the position of the interference fringes is the relative phase between the two peaks of the atomic wavefunction located at the two slits. Each part of the atom interacts with a separate cavity, and as the longitudinal displacement induced on the atom by a single cavity is $\pm\lambda_{dB}/4$, the relative displacement by which the two parts of the atomic wavefunction are shifted can be 0 or $\pm\lambda_{dB}/2$ (corresponding to a relative phase shift of 0 or $\pm\pi$). Without some clever measurement on the cavities, the value of the relative displacement cannot be determined, and the interference pattern is washed out. Such measurements were discussed by Scully *et al.*³ and may be regarded as measurements of the relative phase between the fields in the two cavities. This requires that we describe the photon not as having been deposited in one cavity or the other, but as being shared between the two cavities with some relative phase. Atoms that leave the cavities with a given relative phase do exhibit interference; but if the relative phase information is discarded, shifted interference patterns add together, washing out the fringes. *Welcher Weg* information cannot be obtained at the same time as relative phase information.

The connection between a longitudinal displacement and a transverse momentum kick can be understood intuitively by visualizing a rotation of the atomic wavefront. Because the atom's motion is primarily longitudinal, a transverse momentum kick will simply change its direction slightly. This implies a

differential displacement of the atomic wavefront, the displacement being in the longitudinal direction by an amount which varies in proportion to the transverse position. This is effectively the familiar phenomenon of refraction: a position-dependent change in the phase of a wave results in a change in the direction of propagation.

We have shown that the loss of interference from a double slit in the presence of a *welcher Weg* detector is physically caused by momentum kicks, the magnitude of which are determined by the uncertainty principle. We therefore conclude that the principle of complementarity is a consequence of the Heisenberg uncertainty relation. The source of these momentum kicks in the micromaser detector suggested by Scully *et al.*³ is the repeated emission and reabsorption of microwave photons by the atom. $\square$

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Formation of carbon films on carbides under hydrothermal conditions

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CARBON films find applications in a wide range of fields, ranging from microelectronics to materials science¹. In ceramic matrix composites they confer the high strength and toughness needed for applications in aerospace, nuclear and automotive engineering². Chemical vapour deposition is traditionally used to prepare carbon films, but it is relatively expensive, and not easily adapted to coating samples in the form of whiskers, platelets or powders. Here we report that silicon carbide, the most common component of composite ceramics, can be coated with carbon films of nanometre to micrometre thickness by hydrothermal treatment at 300–800 °C. We have applied the technique to SiC fibres, powders, platelets and single crystals, as well as to other carbides. Our method should provide a general and inexpensive route to high-toughness composites and lubricating coatings.

Chemical vapour deposition at ~1,100 °C (refs 3, 4) is usually used to obtain carbon layers. Interfacial carbon layers have also been formed by long-term, high-temperature treatment of an SiC-containing composite². However, the former method is relatively expensive, complicated and results in a decrease of

strength; the latter is hard to control. It has been reported that a ~100-nm layer of carbon at the Nicalon fibre–matrix interface improves greatly the mechanical strength of glass–ceramic matrix composites⁵. A carbon layer mitigates also the high-temperature degradation of the SiC fibres^{3,4}, which occurs usually during processing or use of the composites above ~1,200–1,300 °C.

Recently, hydrothermal reactions have been used successfully as a non-traditional way of producing powders, single crystals and coating films⁶. For example, it has been shown that hydrothermal treatment at 900 °C and 1,000 MPa water pressure can enhance graphitization of carbon⁷. The substantial solubility of carbon in C–H–O–(Si) fluids, and the possibility of graphite deposition from these fluids have been known for years^{8–11}. The behaviour of powdered¹² and sintered SiC (ref. 13), as well as SiC (Tyranno) fibres¹⁴ under hydrothermal conditions in the temperature range 300–800 °C and pressures up to 100 MPa has been studied, but formation of only silica and gaseous compounds (CO, CH₄) was reported.

We have attempted to adapt the hydrothermal technology to SiC fibres. The important feature of the suggested method is that the film was not deposited on the surface from a solution, but the surface layer of the substrate was transformed into carbon.

To demonstrate the idea, two commercially available (UBE Industries Ltd., Yamaguchi, Japan) amorphous SiC (Tyranno) fibres with the thickness of ~11 µm were chosen. Tyranno fibre is a type of silicon carbide fibre containing titanium as well as oxygen, made by pyrolysis of an organometallic polymer precursor¹⁵. Standard fibre (grade S), and a new fibre (LoxM grade) were used for the experiments. The properties and composition of materials used are described in ref. 14. Density of the fibres was 2.35–2.40 g cm⁻³. The surface of as-received fibres was smooth and featureless. All fibres were X-ray amorphous, and uniform and circular in shape.

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The structure of Tyranno fibre is finer than that of Nicalon¹⁶, and consists of SiC microcrystals, which are connected by Si-O-C and Ti-O (Ti-C-O) interlayers. Taking into account a larger amount of oxycarbide in comparison with SiC in this fibre, we may consider the structure of the fibre as an amorphous SiO_xC_y matrix with nanometre-size β -SiC inclusions. Hydrogen content was less <0.1%.

The fibres (50 mm long) were cleaned in hot water to remove a thin polyethylene oxide (PEO) film from their surface (desized), and placed with distilled water into an Au capsule 60 mm long and 5 mm in diameter. The capsule was heated in a test-tube-type pressure vessel under the pressure (100 MPa) of water vapour. The hydrothermal equipment is described in more detail elsewhere¹⁷. In these treatments, the temperature was kept at 300–600 °C for 25 h.

Scanning electron microscopy (SEM) investigations demonstrated that the outer surface of the fibres heated in water at temperatures of 300–450 °C remained smooth and almost featureless (Fig. 1). Only slight surface damage was sometimes found after treatment at ~450 °C, but surface films with a thickness of 1–2 μ m delaminated under applied load due to mismatch of elastic properties. Spallation of the surface scale was found at higher temperatures.

According to X-ray diffraction, crystallization of the fibres did not occur after hydrothermal treatments in the temperature range under study and the fibres remained amorphous. We expected to find glassy silica in the surface layer of the treated fibres. However, Fourier transform infrared (FTIR) spectroscopy showed that the absorption due to Si-O stretching (1,080 cm⁻¹) became weaker after treatment at 300–400 °C. The absorption at 820 cm⁻¹ due to Si-C stretching disappeared above 500 °C. Another interesting feature of FTIR spectra from the treated fibres was the appearance of absorptions corresponding to hydrogenated carbon. Similar spectra were obtained for amorphous hydrogenated carbon films¹⁸. Auger electron spectroscopy (AES) depth analysis of the surface layer of the fibres (Fig. 2) showed that a carbon layer, containing traces of silicon, was formed after hydrothermal treatment under conditions of our experiment. The thickness of this layer varied from 10–20 nm to 1–2 μ m depending on temperature and time. Raman spectroscopy investigations confirmed the formation of amorphous or microcrystalline carbon (Fig. 3). Calculations¹⁹ demonstrated that the dimensions of crystallites along the basal graphite planes L_a was <5 nm even at the highest treatment temperature. This was confirmed also by transmission electron microscopy (TEM).

It has been shown¹² that hydrothermal corrosion of SiC in the temperature range of interest occurs by the reactions



But our observations demonstrate that the reaction

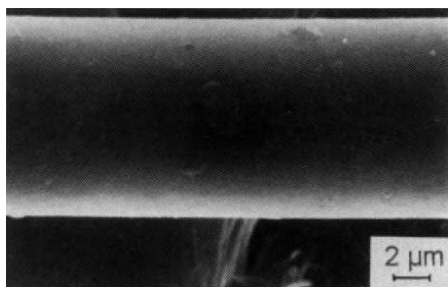
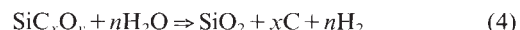


FIG. 1 Scanning electron micrograph showing the morphology of the carbon film on the surface of LoxM-grade Tyranno fibre after hydrothermal treatment at 450 °C. Film thickness is ~2 μ m. The film is dense, smooth and of uniform thickness.

dominates. The possibility of this reaction has been confirmed by SOLGASMIX-type²⁰ thermodynamic calculations (N. S. Jacobson, personal communication). Silica formed was dissolved in water during the treatment. A dissolution rate of ~0.8 μ m h⁻¹ was reported for silica glass in distilled water at 285 °C under 100 MPa pressure²¹. This rate is sufficient for the complete dissolution of silica formed according to reaction (3). AES did not show the presence of oxygen in the surface carbon film at 400–450 °C. If the samples are encapsulated in a sealed container^{12,14}, some silica can remain on the surface of SiC.

As discussed above, Tyranno fibre consists mainly of SiC microcrystals and SiC_xO_y. It contains also C-C bonds, as was found from the Raman spectra of untreated fibres (Fig. 3). Thus, the reaction



as well as free carbon in the fibre can be responsible for the formation of the carbon film. Deposition of carbon from a C-H-O-Si fluid formed due to reactions (1 and 2) is also quite possible⁸. To prove the possibility of reaction (3), we investigated hydrothermal behaviour of an SiC powder (A10, H. C. Starck Co., Berlin), α (6H)-SiC single crystals and α -SiC platelets. In all cases carbon was formed, and Raman spectra similar to that shown in Fig. 3 were obtained. It should be noted that the decomposition of pure SiC occurred at slightly higher temperatures (600–800 °C). Therefore, relatively sharp lines corresponding to microcrystalline graphite (G and D in Fig. 3) were observed on the Raman spectra.

Tensile testing of Tyranno monofilaments according to the ASTM D3379-75 standard with a 25-mm gauge length demonstrated that their strength was not changed after hydrothermal treatment for 25 h at 300 °C, when a film $\leq$ 50 nm thick was formed. Treatment of the LoxM-grade fibre at 400 °C led to a decrease of both strength (3.4 to 2.1 GPa), and Young's modulus (192 to 137 GPa). For the S-grade, strength dropped from 4 to 1.54 GPa and the Young's modulus reduced from 160 to 94 GPa. This decrease in strength was caused by the formation of 1–2- μ m-thick carbon films, which led to a substantial decrease of the cross-section of the fibre. But even in this case, the fibres retained a higher strength than after coating with CVD carbon film of the same thickness⁴ and their thickness decreased slightly, rather than increased as in the case of CVD, which is very important for producing high-strength ceramic composites with small flaw

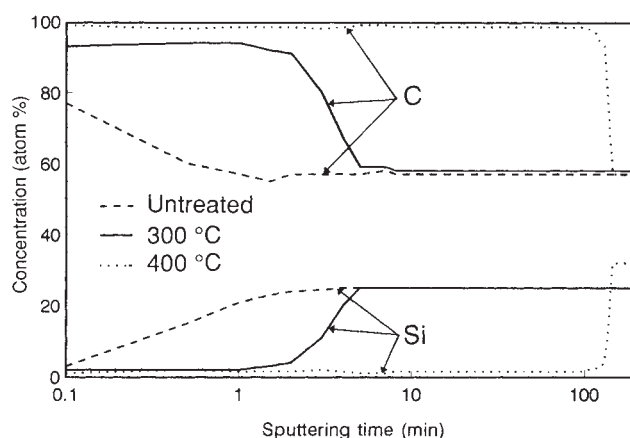


FIG. 2 Auger electron spectroscopy (AES) depth profiles of C and Si (semilogarithmic plot) in the surface layer of S-grade Tyranno fibres before and after hydrothermal treatment for 25 h at 300 °C and 400 °C. The difference from 100 atom % is due to oxygen, which is not shown. The thickness of the carbon film is ~50 nm and 1.4 μ m after the treatments at 300 °C and 400 °C respectively.

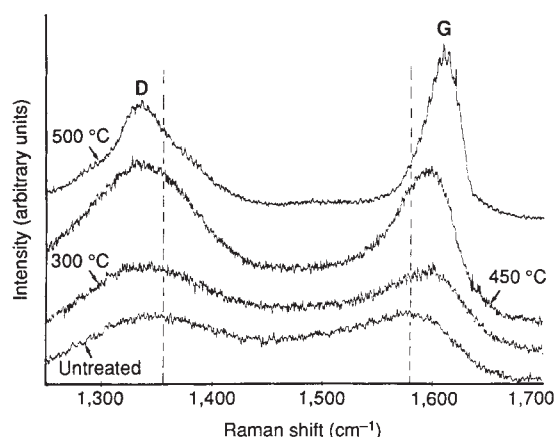


FIG. 3 Raman spectra of the LoxM-grade Tyranno fibres before and after hydrothermal treatments at various temperatures. Ar laser radiation at a wavelength of 488 nm was used. The spectra are composed of a graphite line (G) and a disorder-induced line (D). Vertical dashed lines show the predicted positions of G and D lines for polycrystalline graphite. The shift of the observed lines from 1,580 and 1,355 cm^{-1} indicates the presence of the bond-angle disorder. The up-shift of the G line towards the high-frequency edge means that the crystallites have a very small grain size, and that they are dominated by sp^2 C-C rather than sp^3 C-C bonds²². However, the down-shift of the D line indicates the presence of sp^3 carbon bonds as well as disorder. The decrease of the full width at half maximum of both lines with increasing temperature means the removal of the bond-angle disorder and the increasing dominance of crystallites.

sizes. Calculations of the strength of the SiC core of the fibre with film demonstrated that it was not changed after hydrothermal treatment below 450 °C.

The hydrothermal method has also potential for fabrication of amorphous carbon or graphite films on various silicon carbide substrates. Graphite films can be useful for controlling the friction between SiC and a counterbody in wear applications. Coatings on whiskers and platelets may gradually improve the fracture toughness and work of fracture of ceramic matrix composites. Treatment of fine SiC powders can produce carbon-coated particles which undergo sintering more readily. The presence of a carbon film on the surface allows the electrical resistivity of Tyranno fibres to be varied from 1 to $10^6 \Omega \text{ cm}$, making possible their use in resistive cables. Hydrothermal treatment for longer times and/or at higher temperatures, that leads to full consumption of the fibre core, results in the formation of very thin, hollow carbon pipes with an outer diameter of $<10 \mu\text{m}$ and wall thickness of $\sim 3 \mu\text{m}$. Such carbon structures might be of interest for specific applications such as catalysis. Further investigation of the properties of the carbon films is necessary, however, to identify their ideal applications. Preliminary experiments with TiC, TaC, WC and NbC show that the surface modification of other carbides by formation of carbon, or carbon containing, layers is also possible. □

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Total synthesis of taxol

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TAXOL^{1–4}, a substance originally isolated from the Pacific yew tree (*Taxus brevifolia*) more than two decades ago, has recently been approved for the clinical treatment of cancer patients. Hailed as having provided one of the most significant advances in cancer therapy⁵, this molecule exerts its anticancer activity by inhibiting mitosis through enhancement of the polymerization of tubulin and consequent stabilization of microtubules⁶. The scarcity of taxol and the ecological impact of harvesting it have prompted extensive searches for alternative sources including semisynthesis, cellular culture production and chemical synthesis^{2,3}. The latter has been attempted for almost two decades, but these attempts have been thwarted by the magnitude of the synthetic challenge. Here we report the total synthesis of taxol by a convergent strategy, which opens a chemical pathway for the production of both the natural product itself and a variety of designed taxoids.

The strategy for the present synthesis of taxol (1, Fig. 1a) was based on a retrosynthetic analysis involving the bond disconnections⁷ shown in Fig. 1b. Thus, in the synthetic direction the following key operations were proposed: (1) two fragments, representing precursors to rings A and C (see Fig. 1a), were to be coupled by a Shapiro reaction⁸ and a McMurry coupling⁹ to assemble the ABC ring skeleton; (2) instalment of the oxetane ring; (3) addition¹⁰ of the various substituents around the peripheries of rings B and C; (4) oxygenation¹⁰ at C-13; and (5) esterification to attach the side chain¹¹.

The previously reported intermediates 2 (ref. 12) (Fig. 2) and 8 (refs 7, 13) (Fig. 3) served as the starting points for the convergent synthesis of taxol reported here. Figure 2 presents the construction of the requisite C-ring aldehyde 7 from 2. Protection of both hydroxyl groups in 2 with TBS groups (95%) (for abbreviations see figure legend) followed by selective reduction of the ester group with LiAlH_4 at 0 °C, furnished primary alcohol 3 (94% yield). Acid-catalysed deprotection of the secondary alcohol in 3 proceeded in a highly selective manner to give the corresponding diol (90% yield), which was then selectively protected with a TPS group at the primary position and a benzyl group at the secondary to afford compound 4 in 80% overall yield. The γ -lactone in 4 was then reductively opened with concomitant desilylation at the tertiary position using LiAlH_4 at

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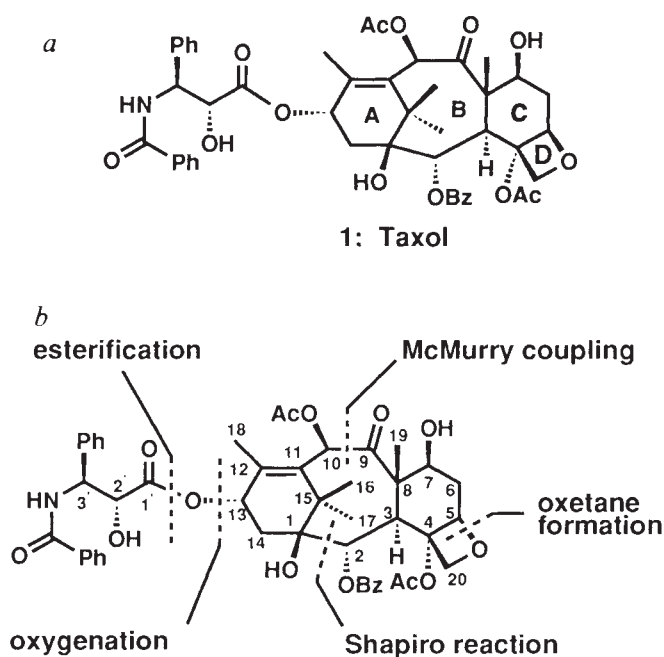


FIG. 1 Structure (a) and strategic bond disconnections of taxol (b). Abbreviations for chemical groups (see also Figs 2, 3 and 5): Ph, phenyl; OBz, benzoyl; OAc, acetyl.

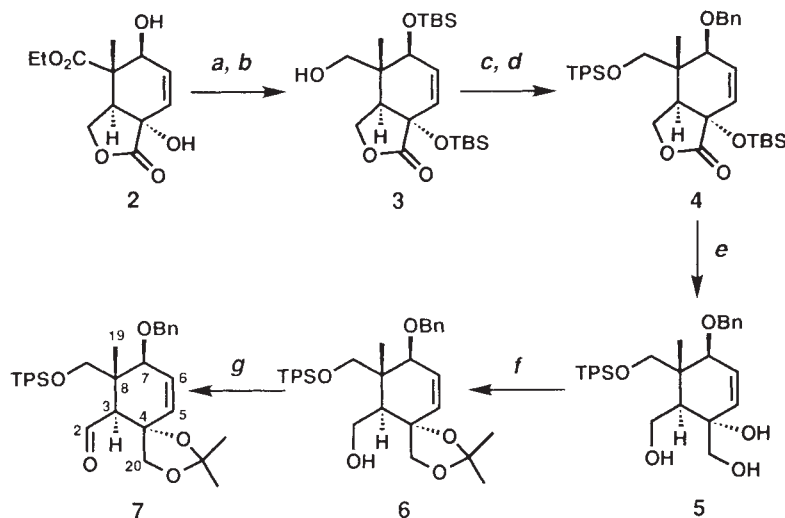
25 °C to produce triol **5** in 80% yield. Finally, acetonide formation followed by TPAP¹⁴ oxidation in the presence of NMO resulted in the formation of the targeted aldehyde **7** in 80% overall yield.

Figure 3 summarizes the coupling of intermediates **7** and **8** and elaboration of the coupling product to give the requisite tricyclic system **13**. When the vinyl lithium reagent derived from aryl hydrazone **8** and *n*-C₄H₉Li (refs 8, 13) was reacted with aldehyde **7** at -78 °C, a single diastereoisomer of hydroxy-compound **9** was obtained in 82% yield. Directed epoxidation of the C1-C14 double bond in **9** was realized, in 87% yield,

using *t*-C₄H₉OOH in the presence of VO(acac)₂ (ref. 15), leading selectively to epoxide **10** which was regioselectively opened with LiAlH₄ to give the 1,2-diol **11** (76% yield). X-ray crystallographic analysis of this compound (**11**) confirmed the designated stereochemistry for intermediates **9–11** and their relatives (Fig. 4a). To prepare the molecule for closure of the 8-membered B ring, and in order to create subsequent opportunities for the introduction of the benzoate functionality at C-2, diol **11** was converted to its cyclic carbonate by exposure to phosgene in the presence of KH, furnishing dialdehyde **12**, after desilylation (*n*-(C₄H₉)₄NF) and oxidation (TPAP-NMO)¹⁴ in 32% overall yield. The suitably preorganized dialdehyde **12** was then subjected to a McMurry-type^{9,13} cyclization to afford the taxoid ABC ring system **13** in 23% yield (stereochemistry at the newly generated centres assigned by X-ray crystallographic analysis of a subsequent intermediate, **13'**; see below and Fig. 4c).

The next important intermediate in the synthesis was **19**, a compound that was reached from **13** as outlined in Fig. 5. Monoacetylation of **13** followed by oxidation with TPAP-NMO¹⁴ furnished, regioselectively in 88% overall yield, ketoacetate **14**. The stereochemistry of the acetate group at C-10 was confirmed through conversion of **14** to the crystalline benzoate **14'** (PCC, NaO(CO)CH₃, celite, benzene, heat) and X-ray crystallographic analysis on the latter (see ORTEP drawing, Fig. 4b). Hydroboration of compound **14** followed by basic hydrogen peroxide treatment led to a mixture of two regioisomeric alcohols (55%, ~3:1 by ¹H NMR) which was subjected to acid-induced removal of the acetonide group and chromatographic separation to afford triol **15** (33% yield from **14**) as the major product. The primary hydroxyl group in **15** was then selectively acetylated under standard conditions, furnishing compound **16** in 95% yield. At this stage the benzyl protecting group on the C-7 oxygen was replaced by a triethyl silyl group (TES) for reasons arising from later stages of the synthesis, and the resulting compound was selectively monodeacetylated under mildly basic conditions (K₂CO₃-CH₃OH) leading to triol **17** (78% overall yield). The oxetane ring was finally constructed by sequential monosilylation with TMSCl (primary OH), triflate formation (secondary OH) and mild acid treatment to afford, after acetylation of the remaining tertiary hydroxyl group, the targeted intermediate **19** in 38% overall yield¹⁶. Racemic **19**, obtained from this sequence, was identical in all respects (except for optical rota-

FIG. 2 Construction of C-ring system **7**. Reagents and conditions. (a) *t*-BuMe₂SiOTf (4 equivalents (eq.)), 2,6-lutidine (4 eq.), 4-DMAP (0.01 eq.), CH₂Cl₂, 0 °C, 4 h, 95%; (b) LiAlH₄ (1.1 eq.), Et₂O, 0 °C, 1 h, 94%; (c) (1) CSA (0.05 eq.), MeOH, CH₂Cl₂, 25 °C, 1 h, 90%; (2) *t*-BuPh₂SiCl (1.5 eq.), imidazole (1.6 eq.), DMF, 25 °C 6 h, 92%; (d) KH (1.2 eq.), Et₂O, *n*-Bu₄NI (cat.), BnBr (1.2 eq.), 25 °C, 2 h, 87%; (e) LiAlH₄ (3 eq.), Et₂O, 25 °C, 12 h, 80%; (f) 2,2-dimethoxypropane (5 eq.), CSA (0.1 eq.), CH₂Cl₂, 25 °C, 7 h 82%; (g) TPAP (0.05 eq.), NMO (1.5 eq.), CH₃CN, 25 °C, 2 h, 95%. (Bn = CH₂Ph; CSA = (±)-camphorsulphonic acid; 4-DMAP = 4-dimethylaminopyridine; DMF = N,N-dimethylformamide; NMO = N-methylmorpholine-N-oxide; TBS = *t*-BuMe₂Si; TPAP = tetra-*n*-propylammonium perruthenate; TPS = *t*-BuPh₂Si.) Selected physical data for compound **7**: ¹H NMR (500 MHz, CDCl₃, taxol numbering): δ 9.98 p.p.m. (d, *J* = 3.5 Hz, 1 H, 2-H), 7.65–7.12 (m, 15 H, aromatic), 5.84 (dd, *J* = 10.5, 1.5 Hz, 1 H, 6-H), 5.71 (dd, *J* = 10.5, 2.0 Hz, 1 H, 5-H), 4.50 (d, *J* = 11.5 Hz, 1 H, OCH₂Ph), 4.22 (d, *J* = 11.5 Hz, 1 H, OCH₂Ph), 4.20 (d, *J* = 9.5 Hz, 1 H, 20-H), 4.10 (dd, *J* = 2.0, 1.5 Hz, 1 H, 7-H), 3.84 (d, *J* = 9.5 Hz, 1 H, 20-H), 3.72 (d, *J* = 10.0 Hz, 1 H, 9-H), 3.70 (d, *J* = 10.0 Hz, 1 H, 9-H), 3.18 (d, *J* = 3.5 Hz, 1 H, 3-H), 1.42 (s, 3 H, CH₃-acetonide), 1.39 (s, 3 H, CH₃-acetonide), 1.09 (s, 9 H, (CH₃)₃CSi), 1.04 (s, 3 H, 19-CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 202.3, 138.1, 135.8, 135.6, 133.0, 132.9, 131.1, 129.7, 129.4, 129.5, 128.8, 128.2, 127.6, 127.4, 127.4, 127.2, 127.1, 108.6, 80.6, 75.4, 71.8, 70.0, 65.7, 57.6, 44.9, 26.9, 26.8, 26.5, 19.3, 13.6; infrared (pure compound): ν_{max} 2,931.4, 2,857.0,



1,720.4, 1,111.5 cm⁻¹; high resolution mass spectrometry (fast atom bombardment) (HRMS (FAB)): calcd for C₃₆H₄₄O₅Si (M⁺ + Cs) mass-to-charge ratio *m/z* = 607.2856 atomic mass units (a.m.u.), found 607.2865 a.m.u.

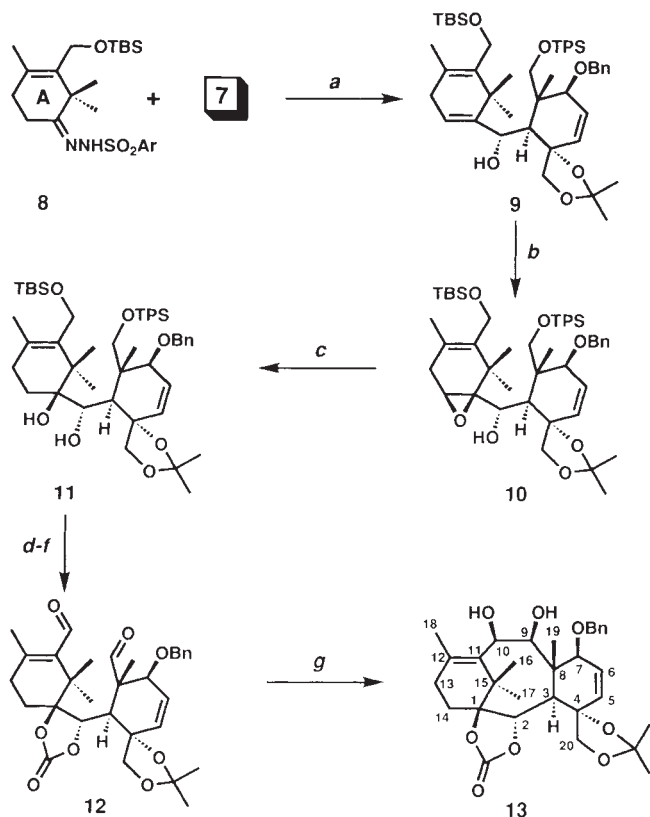


FIG. 3 Construction of ABC ring system 13. Reagents and conditions. (a) (1) **8**, *n*-BuLi (2.05 eq.), THF, $-78^{\circ}\text{C} \rightarrow 25^{\circ}\text{C}$, cool to 0°C and add **7** (1.0 eq. in THF), 0.5 h, 82%; (b) VO(acac)₃ (0.03 eq.), *t*-BuOOH (3 eq.), 4-Å molecular sieve (cat.), benzene, 25°C , 12 h, 87%; (c) LiAlH₄ (3 eq.), Et₂O, 25°C , 7 h, 76%; (d) KH (3 eq.), HMPA/Et₂O (30/70), COCl₂ (20% in benzene, 2 eq.), 25°C , 2 h, 48%; (e) TBAF (10 eq.), THF, 25°C , 7 h, 80%; (f) TPAP (0.05 eq.), NMO (3 eq.), CH₃CN/CH₂Cl₂ (2:1), 25°C , 2 h, 82%; (g) (TiCl₃)₂-(DME)₃ (1.0 eq.), Zn-Cu (20 eq.), DME, 70°C , 1 h, 23%. Ar = 2,4,6-triisopropylbenzene sulphonyl; HMPA = hexamethylphosphoric triamide; NMO = 4-methylmorpholine-N-oxide; TBAF = tetra-*n*-butylammonium fluoride; TBS = *t*-BuMe₂Si; TPAP = tetra-*n*-propylammonium perruthenate. Selected physical data for compound **13**: ¹H NMR (500 MHz, CDCl₃, taxol numbering): δ 7.42–7.31 (m, 5 H, aromatic), 5.97 (dd, $J=10.0, 1.5$ Hz, 1 H, 5-H), 5.63 (dd, $J=10.0, 1.5$ Hz, 1 H, 6-H), 5.46 (d, $J=5.0$ Hz, 1 H, 2-H), 4.77 (d, $J=12.0$ Hz, 1 H, OCH₂Ph), 4.49 (d, $J=8.5$ Hz, 1 H, 20-H), 4.39 (d, $J=12.0$ Hz, 1 H, OCH₂Ph), 4.29 (d, $J=5.5$ Hz, 1 H, 10-H), 4.24 (d, $J=5.5$ Hz, 1 H, 9-H), 3.80 (d, $J=8.5$ Hz, 1 H, 20-H), 3.58 (b, 1 H, 7-H), 2.75–2.71 (m, 1 H, 13-H), 2.61–2.50 (m, 1 H, 13-H), 2.34 (d, $J=5.0$ Hz, 1 H, 3-H), 1.98–1.92 (m, 1 H, 14-H), 1.83–1.74 (m, 1 H, 14-H), 1.58 (s, 3 H, 18-CH₃), 1.45 (s, 3 H, 19-CH₃), 1.42 (s, 3 H, CH₃-acetonide), 1.41 (s, 3 H, CH₃-acetonide), 1.19 (s, 3 H, 16-CH₃), 1.08 (s, 3 H, 17-CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 153.9, 139.4, 137.3, 136.1, 135.6, 128.7, 128.5, 128.3, 122.0, 108.2, 93.4, 82.4, 77.9, 75.7, 74.2, 71.2, 70.4, 69.3, 46.3, 44.3, 40.0, 31.2, 29.6, 28.9, 27.9, 26.8, 23.6, 21.7, 21.3, 16.0; infrared (pure compound): ν_{max} 2,970.3, 1,789.1, 1,455.6, 1,100.3 cm⁻¹; HRMS (FAB) calcd for C₃₁H₄₀O₈ (M⁺ + Cs), m/z = 673.1778 a.m.u., found 673.1782 a.m.u.

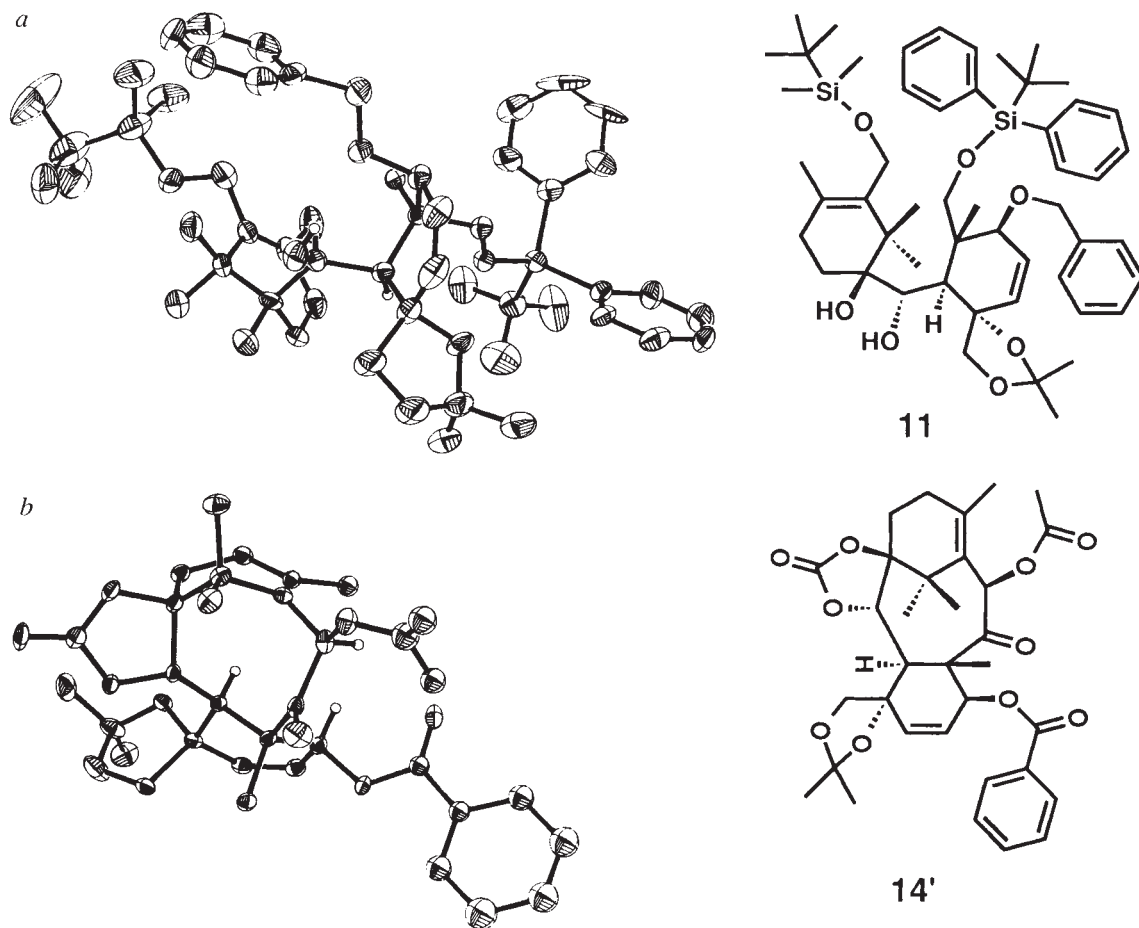
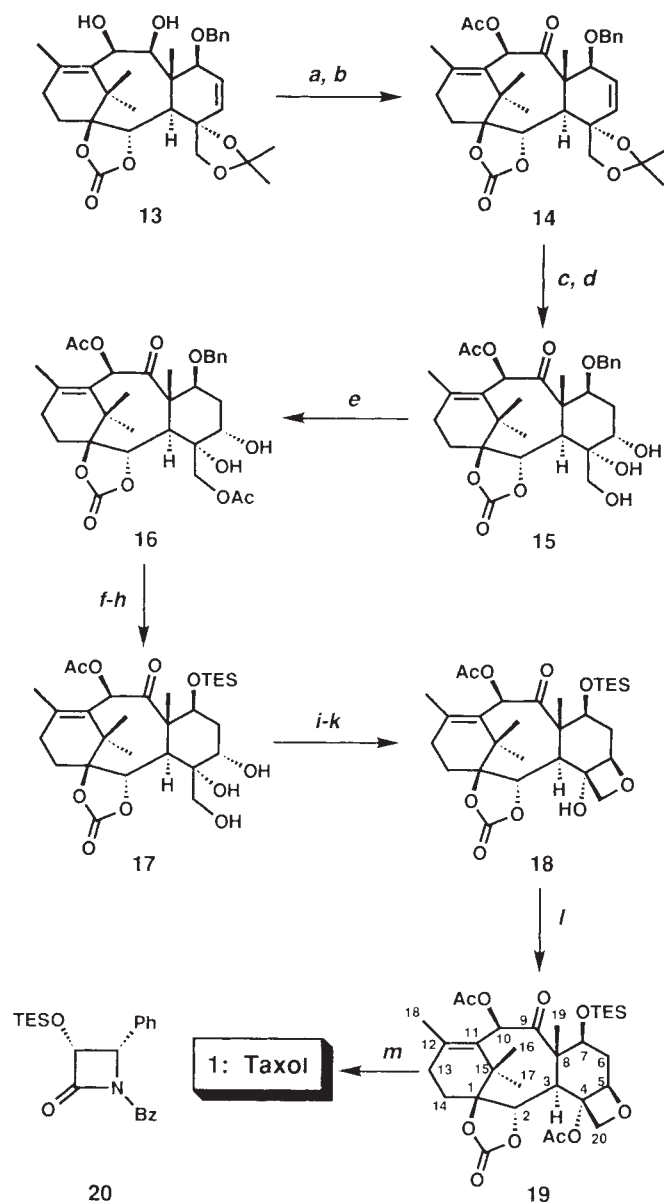


FIG. 4 ORTEP drawings of compounds **11** (a), **14'** (b) and **13'** (c). (ORTEP, Oregon national thermal ellipsoid program.)

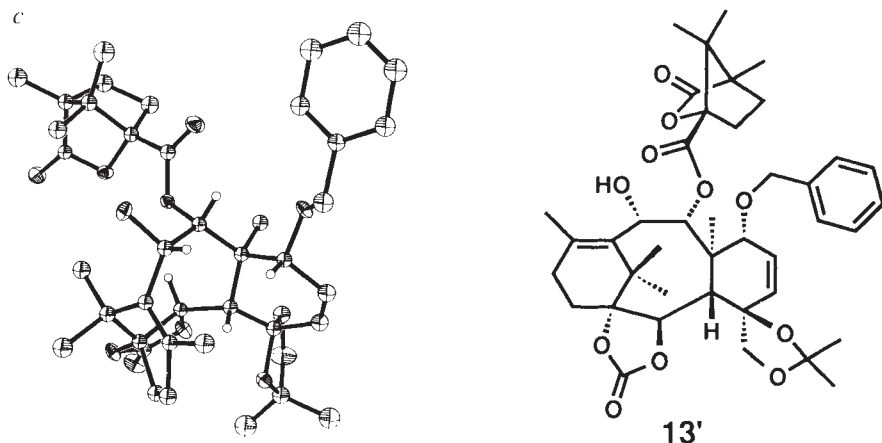
FIG. 5 Total synthesis of ABCD ring system **19** and taxol (**1**). Reagents and conditions. (a) Ac₂O (1.5 eq.), 4-DMAP (1.5 eq.), CH₂Cl₂, 25 °C, 2 h, 95%; (b) TPAP (0.1 eq.), NMO (3 eq.), CH₃CN, 25 °C, 2 h, 93%; (c) BH₃–THF (5.0 eq.), THF, 0 °C, 2 h then H₂O₂, aqueous NaHCO₃, 0.5 h, 55% (~3:1 mixture of C5–C6 regioisomers by ¹H NMR); (d) conc. HCl, MeOH, H₂O, 25 °C, 5 h 80%; (e) Ac₂O (1.5 eq.), 4-DMAP (1.5 eq.), CH₂Cl₂, 25 °C, 0.5 h, 95%; (f) H₂, 10% Pd(OH)₂(C), EtOAc, 25 °C, 0.5 h, 97%; (g) Et₃SiCl (25 eq.), pyridine, 25 °C, 12 h, 85%; (h) K₂CO₃ (10 eq.), MeOH, 0 °C, 15 min., 95%; (i) Me₃SiCl (10 eq.), pyridine (30 eq.), CH₂Cl₂, 0 °C, 15 min., 96%; (j) Tf₂O (15 eq.), *i*-Pr₂NEt (30 eq.), CH₂Cl₂, 25 °C, 0.5 h 70%; (k) CSA (cat.), MeOH, 25 °C, 10 min., then silica gel, CH₂Cl₂, 25 °C, 4 h, 60%; (l) Ac₂O (10 eq.), 4-DMAP (20 eq.), CH₂Cl₂, 25 °C, 4 h, 94%; (m) (1) PhLi (5 eq.), THF, –78 °C, 10 min., 80%; (2) PCC (30 eq.), NaOAc, celite, benzene, reflux, 1 h, 75%; (3) NaBH₄ (10 eq.), MeOH, 25 °C, 5 h, 83%; (4) NaN(SiMe₃)₂ (3.5 eq.), β-lactam **20**, THF, 0 °C, 87%, based on 90% conversion; (5) HF-pyridine, THF, 25 °C, 1.5 h, 80%. (CSA = (±)-camphorsulphonic acid; 4-DMAP = N-dimethylaminopyridine; NMO = 4-methylmorpholine-N-oxide; TPAP = tetra-*n*-propylammonium perruthenate. Selected physical data for compound **19**: ¹H NMR (500 MHz, CDCl₃, taxol numbering): δ 6.40 (s, 1 H, 10-H), 4.95 (d, *J* = 9.0 Hz, 1 H, 5-H), 4.60 (d, *J* = 9.0 Hz, 1 H, 20-H), 4.47 (d, *J* = 9.0 Hz, 1 H, 20-H), 4.43 (dd, *J* = 10.0, 7.5 Hz, 1 H, 7-H), 4.39 (d, *J* = 5.5 Hz, 1 H, 2-H), 3.36 (d, *J* = 5.5 Hz, 1 H, 3-H), 2.71 (m, 1 H, 13α-H), 2.56 (m, 1 H, 6-H), 2.17 (s, 3 H, OAc), 2.15 (s, 3 H, OAc), 2.12 (m, 1 H, CH₂), 2.07 (s, 3 H, 18-CH₃), 1.97 (m, 1 H, CH₂), 1.88 (m, 2 H, CH₂), 1.78 (s, 3 H, 19-CH₃), 1.23 (s, 3 H, 16-CH₃), 1.17 (s, 3 H, 17-CH₃), 0.88 (t, *J* = 7.5 Hz, 9 H, Si(CH₂CH₃)₃), 0.55 (dq, *J* = 8.0, 3.0 Hz, 6 H, Si(CH₂CH₃)₃); ¹³C NMR (125 MHz, CDCl₃): δ 202.6, 170.3, 169.2, 153.1, 144.0, 130.7, 92.8, 84.0, 80.3, 80.0, 76.4, 76.1, 60.3, 43.5, 38.0, 29.7, 29.4, 25.5, 23.1, 21.9, 21.1, 19.1, 9.8, 6.7, 5.2; infrared (pure compound) *v*_{max} 2,924, 1,814, 1,728, 1,461, 1,372, 1,238 cm^{–1}; HRMS (FAB) calcd for C₃₁H₄₆O₁₀Si (M⁺ + Cs) *m/z* = 739.1915 a.m.u., found 739.1929 a.m.u.



tion) with an authentic sample generated from taxol (**1**) or 10-deacetyl baccatin III (ref. 17) as described elsewhere¹⁰. Optically active **19** was obtained by the same route using enantiomerically pure diol **13** secured by resolution with 1(S)-(–)-camphanic chloride. Thus, reaction of racemic **13** with 1(S)-(–)-camphanic chloride gave, in 86% total yield, two diastereoisomers (**13'** and **13''**) which were chromatographically separated and characterized by X-ray crystallographic analysis on one of them (more polar isomer, silica gel, 15% C₂H₅O(CO)CH₃ in benzene, *R*_F = 0.21) (see ORTEP drawing for **13'**, antipode to desired enantiomer; Fig. 4c). Optically pure **13** ([α]_D²² +187°(CHCl₃, *c* 0.5)) was then generated from the correct diastereoisomer (**13''**, less polar, silica gel, 15% C₂H₅O(CO)CH₃ in benzene, *R*_F = 0.26) by exposure to methanolic K₂CO₃ (90% yield).

The conversion of enantiomerically pure **19** to taxol (**1**) followed the sequence¹⁰: (1) excess C₆H₅Li, –78 °C to regioselectively open the carbonate ring and afford the desired hydroxybenzoate functionality (80%); (2) PCC–NaO(CO)CH₃,

benzene, reflux to introduce a carbonyl group at C-13 (75%); (3) excess NaBH₄–CH₃OH to stereospecifically generate the C-13 hydroxyl group (83%); (4) NaN(Si(CH₃)₃)₂ then Ojima's β-



lactam (**20**)¹¹, 0 °C, to attach the side chain (87% yield based on 90% conversion); and (5) HF-pyridine, to remove the silyl groups (80%). Synthetic taxol was found to be identical in all respects with naturally occurring taxol, including spectroscopic characteristics (¹H and ¹³C NMR, infrared spectroscopy, mass spectra, [α]_D²⁵) and biological activity (microtubule stabilization and cytotoxicity against Molt-4 leukaemia cells).

The chemistry described here not only offers a solution to a formidable synthetic challenge but also opens a completely chemical avenue to taxol, other naturally occurring taxoids and synthetic, designed taxoid derivatives. □

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Model assessment of the role of natural variability in recent global warming

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SINCE the late nineteenth century, the global mean surface air temperature has been increasing at the rate of about 0.5 °C per century^{1–3}, but our poor understanding of low-frequency natural climate variability has made it very difficult to determine whether the observed warming trend is attributable to the enhanced greenhouse effect associated with increased atmospheric concentrations of greenhouse gases^{4,5}. Here we evaluate the observed warming trend using a 1,000-year time series of global temperature obtained from a mathematical model of the coupled ocean–atmosphere–land system. We find that the model approximately reproduces the magnitude of the annual to interdecadal variation in global mean surface air temperature. But throughout the simulated time series no temperature change as large as 0.5 °C per century is sustained for more than a few decades. Assuming that the model is realistic, these results suggest that the observed trend is not a natural feature of the interaction between the atmosphere and oceans. Instead, it may have been induced by a sustained change in the thermal forcing, such as that resulting from changes in atmospheric greenhouse gas concentrations and aerosol loading.

The coupled model⁶ consists of general circulation models of the atmosphere and oceans, and a simple model of land surfaces

(see Fig. 1 for the model description.) This model has been used for other studies of climate variability⁷ and to study the transient response of climate to a gradual increase of greenhouse gases in the atmosphere^{6,8–10}. The model is integrated over a 1,000-yr period, keeping unchanged all thermal forcing factors such as the solar constant, atmospheric carbon dioxide concentration. The initial condition for the integration has realistic seasonal and geographical distributions of sea surface temperature, sea surface salinity and sea ice thickness, with which both atmospheric and oceanic components of the model are nearly in equilibrium.

When the time integration of the model starts from an initial condition, the model state drifts towards its own equilibrium. To reduce this artificial drift from realistic surface conditions, the fluxes of heat and water at the ocean–atmosphere interface are adjusted by amounts which vary seasonally and geographically, but do not change from one year to the next⁶. Because these adjustments are independent of the anomalies of surface temperature and salinity, they neither damp nor amplify these anomalies.

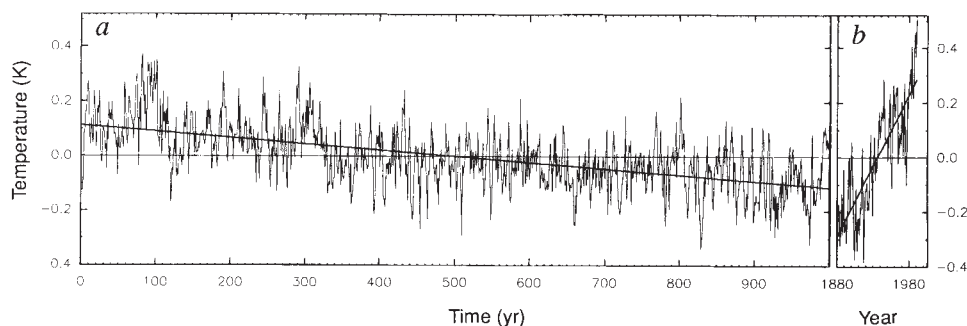
The time series of the globally averaged, annual mean temperature obtained from the 1,000-yr integration (Fig. 1a) has a small trend of –0.023 °C per century. This small linear trend in the time series of global mean surface air temperature of the model is removed in the analysis to follow. This trend is <5% of the trend (0.52 °C per century) in the time series of observed, global mean surface air temperature obtained by Jones and Wigley² (Fig. 1b). In model time series, there are periods during which the rate of the warming trend is as large as 0.5 °C per century, but these periods last only a few decades.

The power spectrum of detrended, globally averaged, annual mean surface air temperature from the 1,000-yr integration of the coupled model is compared to the spectrum of detrended time series of observed temperature² for the period AD 1881–1990 (Fig. 2). The detrending of the observed time series removes most of the contribution from fluctuations on timescales longer than 50 yr. At interdecadal or shorter timescales, the spectrum from the coupled model is not significantly different from the spectrum of observed temperature, although the model estimate is somewhat lower than the observed particularly over the interval of 2 to 5-yr periods. As noted by Lau *et al.*¹¹, this low-resolution model underestimates markedly the magnitude of the Southern Oscillation on interannual timescales. If the contribution from the Southern Oscillation is removed¹² from the detrended observed time series, the standard deviation is 0.101 °C. This compares favourably to the standard deviation (0.096 °C), of the detrended time series from the model. Thus, with the exception of the interannual variations associated with the Southern Oscillation, it appears that the coupled model used here approximately reproduces the observed variability of the global mean temperature on annual to interdecadal timescales.

To assess the probability of finding a century-scale warming trend such as that observed between AD 1881 and 1990 in the time series from the coupled model, we calculated the fraction of occurrence for linear trends (calculated over various time intervals) exceeding 0.5 or 0.25 °C per century (Fig. 3). As the time interval over which the trend is calculated grows longer, the fraction of occurrence becomes smaller. For intervals longer than ~60 yr, there are no trends as large as 0.5 °C per century; for intervals longer than ~90 yr, there are no trends as large as 0.25 °C per century. Therefore the observed warming trend of 0.52 °C per century is not found in the coupled model time series for any intervals longer than ~60 yr. The standard deviation of the linear trend calculated over 110-yr intervals from the detrended time series from the coupled model is ~0.07 °C per century, and is much smaller than the observed warming trend. Although a small trend of 0.023 °C per century is removed from the time series as discussed earlier, the effect of this detrending on the analysis described here is small. In short, it is not very likely that the ocean–atmosphere interaction in the coupled

FIG. 1 Time series of globally averaged, annual mean surface air temperature anomaly from a long-term mean. *a*, 1,000-yr time series from the coupled ocean-atmosphere model. *b*, 110-yr (AD 1881–1990) time series of observed, globally averaged temperature². The straight lines through both time series are such that the sum of squared distance between the time series and the straight line is minimized.

METHODS. The coupled ocean-atmosphere model⁶ has a global domain with realistic geography. The atmospheric component of the model includes seasonally varying insolation, and predicted cloud cover which depends only on the relative humidity. It has nine vertical finite-difference levels. Horizontal distributions of the predicted variables are represented by spherical harmonics (15 zonal waves and 15 associated Legendre functions) and by values at grid points with a 4.5° latitude by 7.5° longitude horizontal spacing. Budgets of heat and water are calculated at the continental surface. It is assumed that continental ice sheets do not change with time. The



oceanic component of the model uses a finite-difference technique with a regular grid which has horizontal spacing of 4.5° latitude by 3.7° longitude, and 12 vertical levels. The model is similar to that of Bryan and Lewis¹⁷, except that the effects of mesoscale eddies are represented by diffusion of potential temperature and salinity on isopycnal surfaces. The atmosphere and ocean interact through the exchanges of heat, water and momentum.

model could randomly generate a substantial long-term warming trend, such as that observed since the end of the last century.

Because the global mean fluctuations are an aggregate of the local fluctuations, it is important that the local fluctuations are realistic in the model's time series. We have therefore compared the geographical distribution of the standard deviation of the time series of local annual mean, detrended surface air temperature from the coupled model with the corresponding distribution for the observed surface air temperature² (Fig. 4*a* and *b*). The standard deviation over continents is usually larger than that over the oceans in both simulated and observed results, with the exception of the tropical eastern Pacific, where the variability of

observed sea surface temperature is relatively large owing to the Southern Oscillation. Nevertheless, it is encouraging that the low-resolution coupled model simulates the gross features in the geographical distribution of the observed interannual variability of surface air temperature.

We also calculated the regression coefficient of local temperature against the global mean temperature in order to assess how the temporal fluctuations of local temperature are related to the fluctuations of the global mean temperature in the model (Fig. 4*c*). The distribution of the regression coefficient is also calculated for the observed surface air temperature anomalies² (Fig. 4*d*) for comparison. In both simulated and observed distributions, the regression coefficient is relatively large over Eurasian and North American continents, although it is exaggerated by the model in the northern part of Eurasia (Fig. 4*c* and *d*). This implies a strong correspondence between the temperature fluctuations over continental regions and the fluctuations of global mean temperature. On the other hand, regression coefficient is near zero (and slightly negative) over the northwestern North

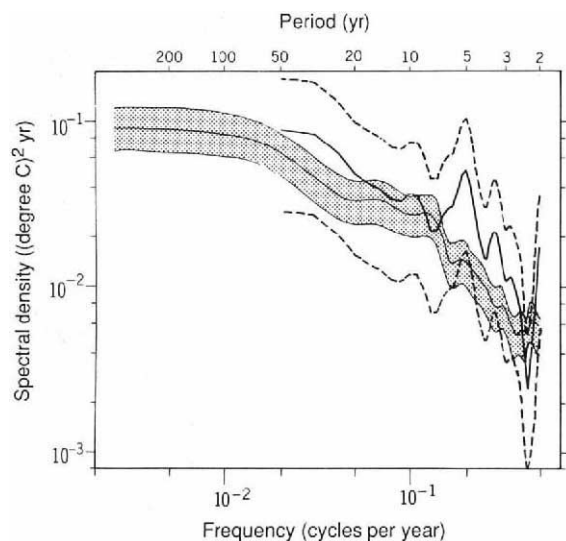


FIG. 2 Power spectra of globally averaged, annual mean surface air temperature. The solid, heavy line denotes the spectral estimates from the detrended, observed data set of Jones and Wigley², and the dashed, heavy lines denote the 95% confidence limit of those estimates. The thin, solid line (within the stippled area) denotes the spectral estimates from the 1,000-yr integration of the coupled model, and the stippled region represents the 95% confidence interval.

METHODS. The spectra for both the observed and model time series were calculated by taking the Fourier transform of the autocovariance function using a Tukey window with a maximum of 30 lags (see Chatfield¹⁸ for details of the spectral estimation and the confidence intervals).

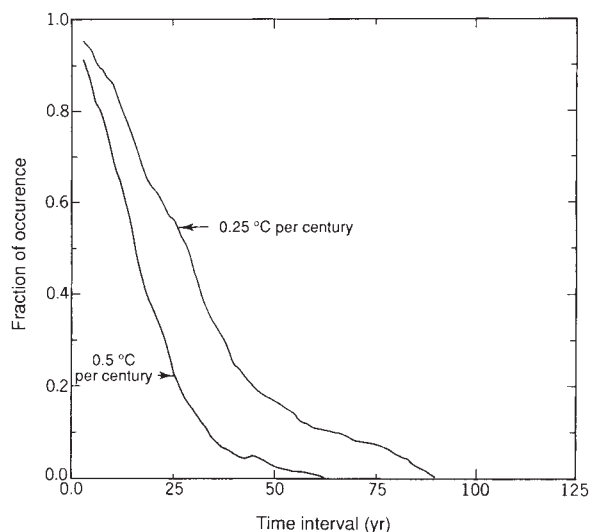


FIG. 3 The fraction of occurrence for the magnitude of the linear trend over a given time interval exceeding 0.5°C per century or 0.25°C per century (solid lines) in the 1,000-yr time series of globally averaged, annual mean surface air temperature of the coupled model. Linear trends are calculated over all combinations of sequential years at various time intervals, using a least-squares technique.

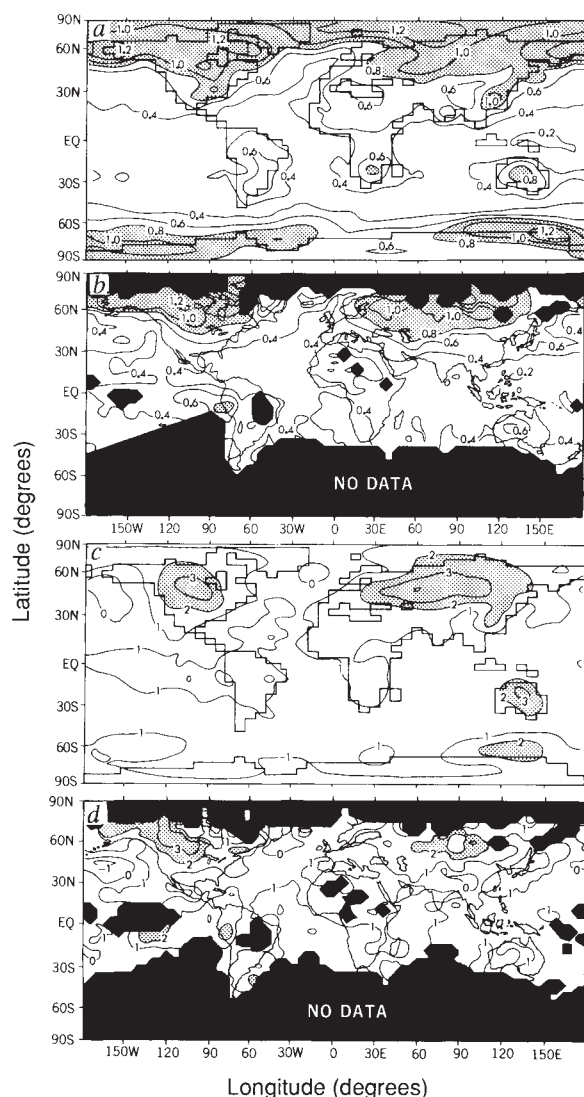


FIG. 4 *a, b*, Geographical distributions of the standard deviation of annual mean surface air temperature (contours, °C) calculated for *a*, the 1,000-yr, detrended time series from the coupled model, and *b*, the 110-yr observed time series compiled by Jones and Wigley². *c, d*, Geographical distributions of the regression coefficient (contours) between the anomalies of local and globally averaged annual mean surface air temperatures from their time mean values calculated for *c*, the detrended 1,000-yr time series from the coupled model, and *d*, the observed time series for the AD 1881–1990 period². Here, for example, a value of 3 implies that for a warming (cooling) of 1 °C in the global mean, the local response is 3 °C warming (cooling). METHODS. The observed, annual mean temperature used here is obtained at those grid points (5° × 5° spacing) where at least ten monthly mean values are available. Both the observed standard deviation and regression coefficients are calculated at those grid points where at least 33 annual mean values are available.

Atlantic and North Pacific in both simulated and observed distributions. A similar pattern of regression coefficient has been obtained from the model separately for interannual or decadal time scales. In summary, the coupled model is able to reproduce reasonably well the correspondence between the fluctuations of the local and global mean temperature.

Other authors have attempted to estimate the internal variability of the atmosphere–ocean system. Hansen *et al.*¹³ have shown that a 100-yr integration of an atmospheric general circulation model coupled with a mixed layer ocean model (instead of the full ocean model used here) exhibits fluctuations in global mean surface air temperature of up to 0.4 °C over a few decades¹⁴. This is similar to the variability found in this present study.

Wigley and Raper¹⁵ evaluated the red-noise-like response of sea surface temperature (from an upwelling diffusion model of the ocean) to a white-noise thermal forcing of the atmosphere. The magnitude of the white-noise forcing is chosen such that the model mimicks the temporal variation of the observed sea surface temperature at relatively high frequencies. They found that the trend of sea surface temperature generated by the model is insufficient to account for the observed warming during the twentieth century, in qualitative agreement with the results from the present study.

As noted by Wigley and Raper¹⁵ and Hasselmann¹⁶, the magnitude of global temperature fluctuations depends significantly

upon the sensitivity of the model climate. The equilibrium response of the global mean, surface air temperature of our model to a doubling of atmospheric CO₂ concentration has been estimated to be ~3.5 °C, in the upper half of the 1.5–4.5 °C range obtained by the IPCC^{4,5}. A model with smaller sensitivity would have smaller variability than our model, making it even less likely to produce the observed warming of the past 110 yr.

It is impossible to judge whether a more realistic model (that is, higher computational resolution, improved parameterization, and so on) would produce a time series which would contain larger trends on century timescales. However, we can not find a trend which is as large and as sustained as the observed 110-yr warming trend of ~0.5 °C per century in the 1,000-yr time series obtained from the coupled model. □

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Target recognition and visual maps in the thalamus of achiasmatic dogs

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VISION is dependent on ordered neuronal representations or maps of visual space. These maps depend on precise connections between retinal axons and their target cells. In mammals, nerve fibres from right and left eyes produce congruent maps of contralateral visual space in adjacent layers of the lateral geniculate nucleus (LGN)¹. We have identified an autosomal recessive mutation in Belgian sheepdogs^{2,3} that eliminates the optic chiasm. In these mutants, all retinal axons project into the ipsilateral optic tract, including those originating in the nasal hemiretina that normally cross midline. These animals exhibit a pronounced horizontal nystagmus^{4,5}. The abnormal ipsilaterally directed nasal fibres innervate the LGN as if they had successfully crossed the midline, terminating in the appropriate layer of the nucleus. As a consequence, the LGN contains non-congruent, mirror-image maps of visual space in adjacent layers. These results show that there is a robust affinity between nasal and temporal retinal axons and specific LGN layers even when all retinal axons originate from a single eye.

Other than the elimination of the optic chiasm, no other midline abnormalities are evident in the mutant dogs. In seven of eight cases, the crossed component of the optic chiasm is absent (Fig. 1). The optic nerves fail to approach midline, and maintain a separation of 5–8 mm as they enter the thalamus. In one animal (M2), a small contralateral retinal projection was apparent (D.H. and R.W., unpublished results).

The shape and lamination of the LGN in the normal dog resembles that of other carnivores. In comparison to the cat, the central field representation in the dog is positioned more rostrally, and the caudal, vertically oriented limb of the nucleus is more prominent (Fig. 2a)^{1,6}. The LGN of mutant dogs closely resembles that of normal dogs in overall size, shape, and cellular composition (Fig. 2b). A prominent interlaminar zone separates layers A and A1 through the central part of the nucleus of

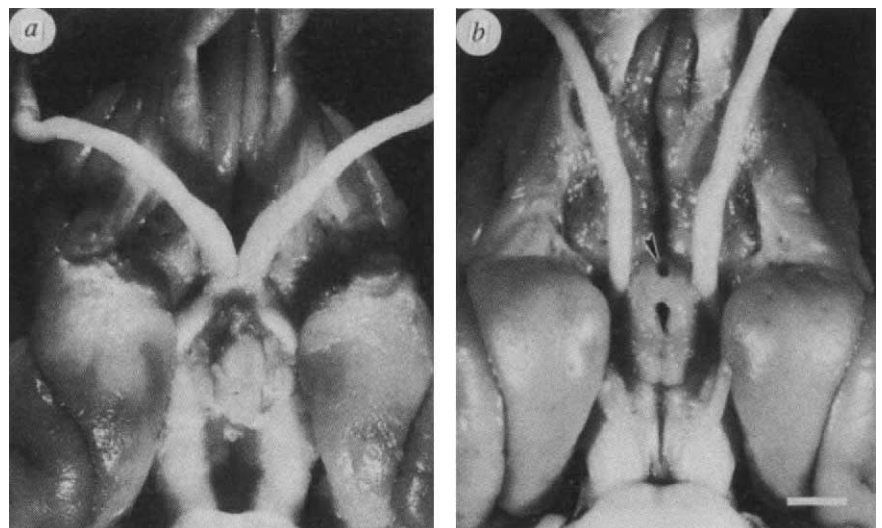
mutants. In contrast to normal dogs, the interlaminar zone does not extend into the rostromedial part of the nucleus. In this area, which represents central visual fields, layers A and A1 are fused (Fig. 2b, c). Upper visual fields are located caudomedially, and lower visual fields are located rostromedially in the LGN of both normal and mutant dogs. The monocular segment of the visual field of normal animals is situated in the dorsocaudal limb of the LGN. As in other carnivores⁷, receptive fields recorded from neighbouring segments of layers A and A1 in normal dogs view the same area of contralateral visual space.

In mutants, as in normal dogs, visual stimuli in the contralateral field evoked vigorous responses in layer A1 (Fig. 3). This relatively small, normal ipsilateral temporal projection is unperturbed by the aberrant ipsilateral input of nasal retina to neighbouring layer A. In each mutant, the entire aberrant nasal projection establishes functional connections throughout ipsilateral layer A. These aberrant ipsilateral fields extended to 90–100° in azimuth and included the region occupied by the contralateral monocular segment of layer A in normal dogs. The misconnection of the nasal retina leads to a severe misalignment of visual maps in layers A and A1. Within a large central segment of the LGN, we repeatedly recorded abrupt reversals in receptive field azimuth (Fig. 3, curved arrows). The size of the reversals varied systematically with the mediolateral position of the penetration. In medial parts of the LGN, small jumps in azimuth were recorded. In more lateral parts of the LGN, large jumps in azimuth were observed (Fig. 3). These reversals across the vertical meridian representations occurred as the electrode crossed the border between A and A1.

The achiasmatic Belgian sheepdog is the first mutant vertebrate in which the size of the ipsilateral retinal projection is increased. Interestingly, achiasmatic humans have also been described recently⁸. The achiasmatic mutation has an effect opposite to that produced by albinism, which increases the size of the contralateral projection. However, the achiasmatic mutation is not a simple anti-albino. In albinism, the line of decussation is shifted towards the temporal edge of the retina and there is invariably a residual uncrossed projection from the far temporal retina^{9–13}. In contrast, in achiasmatic dogs, the phenotype is more extreme because the much larger nasal projection is misrouted and there is usually no residual crossed projection. Although the misrouting of axons in mutant dogs has an opposite pattern to that seen in albinos, the fundamental mapping errors are reminiscent of those observed in the LGN of Siamese cats and albino ferrets^{14–17} (Fig. 4b, c).

The novel pattern of misrouting of retinal fibres in achiasmatic mutants provides a challenging natural experiment that can be used to study processes responsible for generating LGN lamina-

FIG. 1 Ventral views of Belgian sheepdog brains showing *a*, the normal, and *b*, the mutant achiasmatic phenotypes (case M3). *a*, In the normal dog, the two optic nerves meet at midline, and about 80% of the retinal fibres from each nerve cross midline to enter the contralateral optic tract. *b*, In most mutants, the chiasm is eliminated entirely, and all fibres extend into the ipsilateral tract. The minimum distance separating the two retinal projections is ~5 mm. The arrowhead points to the lamina terminalis of case M3. This structure is normally covered ventrally by the optic chiasm. The pituitary has been removed in *b*, exposing the floor of the third ventricle at the midline. Scale bar, 5 mm.



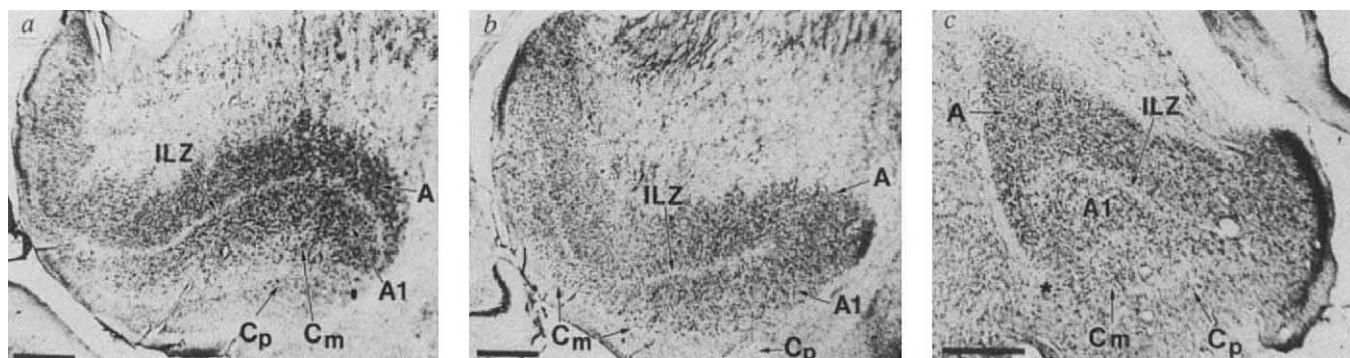


FIG. 2 Lamination of the LGN in normal and achiasmatic mutant dogs. *a*, In this parasagittal plane, the A layer of the LGN of a normal dog appears S-shaped, with its caudal (left) and rostral (right) parts wrapping dorsally and ventrally, respectively. Layer A1 is separated from layer A by a cell-sparse interlaminar zone (ILZ). Note that in the caudal part of the LGN, layer A1 is normally situated caudal to layer A. The magnocellular C layer (C_m) follows the S-shape caudally and extends beneath both layers A and A1 in the rostral, binocular portion of the nucleus. Unlike the situation in cats, there is no interlaminar zone between layer C_m and layer A1 in the dog⁶. The parvocellular C layers (C_p) are located in the most caudal and ventral portions of the LGN. These layers are thinner than C_m and are separated from C_m by a narrow interlaminar zone. *b*, Parasagittal section through the LGN of mutant M3, about

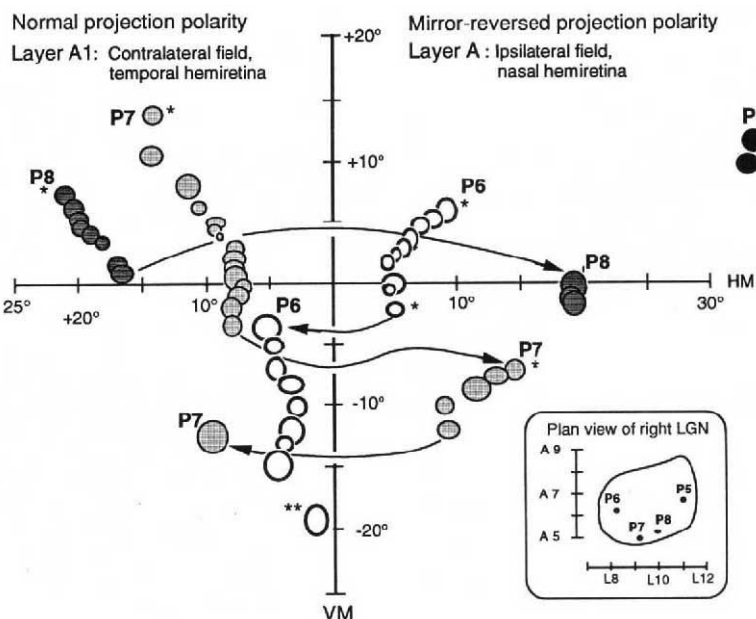
0.5 mm medial to that of the normal dog shown in *a*. The nucleus is similar in overall shape and size to that of normal dogs. However, the interlaminar zone between layers A and A1 is only present in the mid- and caudal portions of the nucleus. In the rostral LGN, layers A and A1 are tightly apposed or fused in mutants. Similar lamination defects may be present in the C layers, but they are not obvious in Nissl-stained material. Cell size does not differ from that of normal animals. *c*, Photomicrograph of a coronal section through the LGN of mutant M1. Fusion of A and A1 is evident on the medial (left) side of the nucleus. The asterisk indicates the location of an electrolytic lesion made along penetration P6 at the junction of A1 with C_m , and corresponds in position to the double asterisk in Fig. 3. Scale bar on each photomicrograph represents 1 mm.

tion and visual maps (Fig. 4). In the achiasmatic dog, a single retina innervates a structure that normally receives input from two retinæ. A single input that projects to a single nucleus might be expected to produce a single map in an un laminated nucleus. This is the pattern found in the LGN after unilateral enucleation early in development^{18, 20} (Fig. 4*d*) and also found normally in another retinal target—the superior colliculus²¹. In fact, the

LGN of mutants is laminated and nasal retinal fibres terminate in ipsilateral layer A with the same anatomical polarity normally seen in the contralateral LGN—the central nasal part of retina projects into the rostral and medial part of the LGN and the peripheral nasal part of the retina projects into the caudal and lateral part of the nucleus. As these fibres have not crossed midline, the physiological polarity of the projection is reversed, both

FIG. 3 Receptive field discontinuities in the achiasmatic mutant. These data are taken from case M1. Note the large receptive field discontinuities (long curved arrows) along each of three penetrations through the LGN (P6–P8). The large, nearly symmetrical shifts in azimuth across the vertical meridian representation (VM) is larger in the more lateral penetrations (P7 and P8). Electrolytic lesions (*) were used to verify that the jumps occurred at the borders between layers. Double asterisk marks a lesion along P6 that is also marked in Fig. 2*c*. Penetrations P7 and P8 were so far caudal in the LGN that the electrode initially traversed layer A1 before finally entering layer A. In contrast, more rostral penetrations initially traversed layer A and then passed into A1. The most lateral penetration (P5) extended through the far peripheral representation that would represent the monocular segment of the LGN in a normal dog. The progression of fields from high to low elevation is normal, and is caused by the dorsoventral orientation of geniculate layers. Small inset is a schematic plan view of the four electrode penetrations. HM, approximate horizontal meridian representation; VM, approximate vertical meridian representation.

METHODS. We recorded from three mutant dogs ranging in age from 3 months to 1 year (M1, M2 and M3; 5–22 kg). As controls, we recorded from one behaviourally normal Belgian sheepdog and three normal adult mongrels (8–24 kg). Dogs were sedated with ketamine hydrochloride (7 mg kg^{-1}) and xylazine (0.4 mg kg^{-1}). Anaesthesia was induced with sodium pentobarbital ($15\text{--}20 \text{ mg kg}^{-1}$). During the course of the experiments, anaesthesia was maintained by infusing sodium pentobarbital ($3 \text{ mg kg}^{-1} \text{ h}^{-1}$). Paralytic agents were not used. Heart rate was monitored. The eyes were protected with contact lenses, stabilized by suturing to eye rings, and focused on a tangent screen 57 cm from the dog's eyes. A craniotomy was made over the thalamus. Low-impedance tungsten microelectrodes ($1 \text{ M}\Omega$) were used to record from visually responsive cells in the LGN. The electrodes were advanced vertically into the LGN. Typically, 3–6 penetrations yielded long sequences of visual receptive fields. The position of the area cen-



tralis and the meridia were estimated with reference to the plotted position of the optic disks (20° central and 5° inferior). Whenever possible, receptive fields were plotted every $100\text{--}200 \mu\text{m}$ along penetrations through the LGN, and small electrolytic lesions were made to facilitate track identification and reconstruction. At the conclusion of the experiment, animals were given an overdose of pentobarbital ($30\text{--}40 \text{ mg kg}^{-1}$; i.v.) and perfused transcardially with fixative. The brain, eyes and optic nerves were dissected free from the cranium. Brains were sectioned at $50 \mu\text{m}$ on a freezing microtome and stained with cresyl violet.

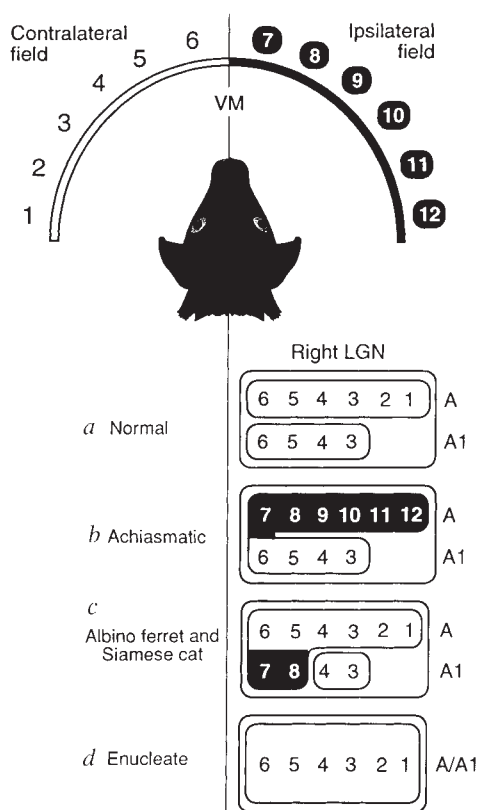


FIG. 4 Simple schemes of lamination and the representation of visual space in layers A and A1 of the carnivore LGN. The horizontal meridian of the visual field is represented as a numbered semicircle. In the schematics of the right LGN, dark areas represent abnormal representations of the ipsilateral visual field. *a*, The pattern of lamination, retinotopy and visuotopy of normal dogs is comparable to that seen in other carnivores. The area of contralateral visual space viewed by the two eyes is aligned in precise register across layers A and A1 (ref. 1). *b*, The achiasmatic pattern in which the entire ipsilateral hemifield (numbers in dark blocks) is represented in layer A. These ipsilateral fields in layer A are out of register with the contralateral fields in layer A1. *c*, The common pattern in the LGN of Siamese cat and albino ferret, in which the lamination abnormality affects only the medial part of layer A1 (refs 14–17). *d*, The pattern following prenatal or early postnatal enucleation in carnivores^{17,19}. Contralateral to the remaining eye, layers A and A1 have formed a composite layer A/A1.

with respect to that in normal dogs and with respect to that in the relatively normal layer A1 of mutant dogs. This results in mirror-image maps in layers A and A1 that cover the entire visual space and which are congruent only at the representation of the vertical meridian (Fig. 4b). It is possible that the vertical meridian is duplicated in A and A1 and that these layers contain independent, but fused maps. It is also possible that there is a single map wrapped around on itself at the vertical meridian representation. The well-ordered mirror-image maps in the mutant LGN indicate that genes that control the crossing of retinal axons at the chiasm differ from those that regulate the formation of maps in the LGN.

Despite its monocular innervation, three relatively normal characteristics of the mutant LGN are surprising. It is not obvious why the so-called binocular and monocular segment of LGN layers would be preserved in the entirely monocular LGN of the mutants (Fig. 2), why nearly symmetrical receptive field positions would be recorded across the A/A1 border (Fig. 3), or why the point at which layers A and A1 are fused would correspond to the vertical meridian. All three features can be explained if positional specificity between retinal axons and LGN cells and layers is rigidly preserved, despite the misrouting of nasal axons at the chiasm. The preservation of target recogni-

tion in mutants most probably reflects a fixed chemoaffinity between subsets of retinal axons and LGN neurons^{22–24}. This mutation shows that the selection of a target site in the LGN is not controlled by the eye of origin (namely, ipsilateral or contralateral), but rather that the critical factors are the retinal position from which axons originate and positional specificity within the LGN. □

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Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness

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MUTATIONS in the gene for the visual pigment rhodopsin cause retinitis pigmentosa (RP) and congenital night blindness^{1–7}. Inheritance of the diseases is generally autosomal dominant and about 40 different rhodopsin mutations have been documented. Although the cell death and retinal degeneration associated with RP have been suggested to result from improper folding and accumulation of the mutant proteins in rod photoreceptor cells⁸, this may not account for the disease in all cases. For example, RP mutations at Lys 296, site of Schiff base linkage to the retinal chromophore, result in constitutive activation of the protein *in vitro*^{9–11}; that is, the mutants can catalytically activate the G protein transducin in the absence of chromophore and in the absence of light. Similarly, mutation of Ala 292 → Glu activates opsin *in vitro* and causes night blindness⁷. We show here that the mutation Gly 90 → Asp (G90D) in the second transmembrane segment of rhodopsin, which causes congenital night blindness¹², also constitutively activates opsin. Furthermore, we show that Asp 90 can substitute for the Schiff base counterion, Glu 113, which is located in the third transmembrane segment of the protein. This demonstrates the proximity of Asp 90 and Lys 296 in the three-dimensional structure of rhodopsin and suggests that the constitutively activating mutations operate by a common molecular mechanism, disrupting a salt bridge between Lys 296 and the Schiff base counterion, Glu 113.

Figure 1 shows that the Gly 90 → Asp (G90D) mutant rhodop-

sin is inactive in the dark and has wild-type activity in the light. In the absence of retinal the opsin form of the mutant is constitutively active. Quantitatively, the activity is less than that observed with A292E (ref. 7), but it is many-fold greater than that for wild-type opsin under identical conditions.

The absorption spectrum of the mutant purified from COS cells after reconstitution with the 11-*cis*-retinal chromophore has a maximum at 483 nm (Fig. 2A), slightly blue-shifted relative to wild-type rhodopsin (500 nm). Upon exposure to light, the mutant undergoes rapid formation of a blue-shifted intermediate, followed by the slow conversion of this intermediate to a 380-nm-absorbing species. Acid-trapping of the 380-nm species¹³ shows that it is free retinal, not bound to protein. The bleaching behaviour of the G90D mutant is very similar to that reported for the A292E mutant⁷. Under similar conditions, wild-type rhodopsin is converted completely to a 380-nm-absorbing species in the dead time of the experiment. The 380-nm species from wild-type is the active intermediate metarhodopsin II (MII; refs 14, 15) and not free retinal because acid-trapping converts it to a 440-nm-absorbing protonated Schiff base. Despite the dramatic difference between the bleaching behaviour of G90D and wild-type, G90D has wild-type ability to activate transducin in the light under our assay conditions (Fig. 1).

Of twelve amino acids substituted at position 296, all except for Lys 296→Arg were constitutively active¹¹. Mutation of the Schiff base counterion, Glu 113, also results in constitutive activation of the apoprotein⁹. These results suggest that in the apoprotein form, opsin is constrained in an inactive conformation partly as a result of a salt bridge between Lys 296 and Glu 113. Mutation of Lys 296 or Glu 113 breaks this salt bridge and promotes transition to the active conformation^{9, 11}. The close proximity of Ala 292 to Lys 296 (one α -helical turn removed from one another) suggests that the mechanism of activation in the A292E mutant also involves disruption of the Glu 113-Lys 296 salt bridge, perhaps by introducing a negative charge that can compete with Glu 113 for the positively charged Lys 296 (ref. 7).

The phenotype of the G90D mutant is similar to that of A292E, so the mechanism of constitutive activation may also be similar. The mutation of a neutral glycine to aspartic acid places a negative charge close to Lys 296 so that it may compete with Glu 113 for the positive charge and disrupt the salt bridge. The problem with this interpretation is that there is no evidence to show that Gly 90 is close to Lys 296 in the three-dimensional structure of rhodopsin.

We have tested this model using the double mutant E113Q, G90D. Mutation of Glu 113 to E113Q causes a dramatic change in the pK_a of the Schiff base nitrogen from >11 in the wild-type to 6 in the mutant^{16, 17}. The Schiff base is therefore not protonated when the protein is isolated at pH 7.4, and the absorption maximum is at 380 nm (Fig. 2B). If Gly 90 lies close to Lys 296, then changing the neutral glycine in the E113Q mutant to aspartic acid might raise the pK_a and restore the protonated state of the Schiff base, shifting the absorption maximum back

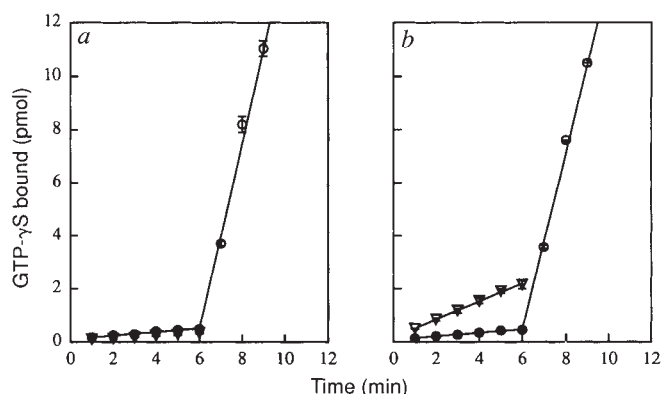


FIG. 1 Activation of transducin by *a*, wild-type rhodopsin, and *b*, the mutant G90D. Δ , Reaction in the absence of 11-*cis*-retinal; $\bullet$, reaction in the presence of 11-*cis*-retinal in the dark; $\circ$, dark reaction after exposure to light at $t = 6$ min.

METHODS. DNA procedures including construction of mutants, were described^{16, 20, 30, 31}. Wild-type opsin and the G90D mutant were assayed in COS cell membranes at pH 6.7 for their ability to activate transducin catalytically by following the binding of [³⁵S]GTP-γS as described^{9, 20}. COS cells were transfected with a synthetic gene³⁰ for wild-type or mutant bovine opsin using DEAE-dextran³¹ and 72 h after transfection were collected and the membranes isolated by sucrose density-gradient fractionation⁹. Membranes were assayed either in the presence or absence of 11-*cis*-retinal at 100 μ M; reactions in the presence of 11-*cis*-retinal were first assayed in the dark and then after exposure to light. Equal amounts of wild-type and mutant opsins were used in the assays as judged by western blot analysis and by purification of the reconstituted pigments from transfected cells. Error bars represent the range of values from two independent and representative determinations of activity.

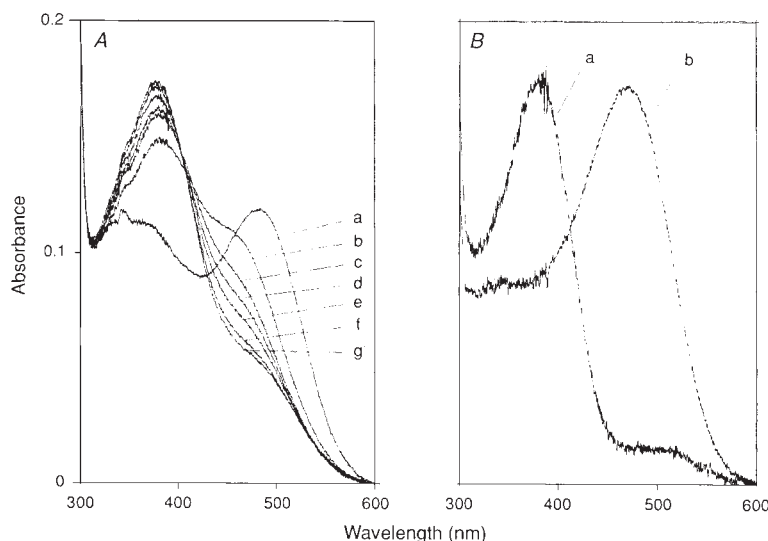


FIG. 2 Absorption spectra of *A*, the G90D single mutant, and *B*, the E113Q, G90D double mutant. For *A*, the G90D mutant was reconstituted with 11-*cis*-retinal and purified from transfected COS cells by an immunoaffinity procedure as described^{16, 31}. The sample was isolated in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.1% dodecylmaltoside. After recording the spectrum (*A*, *a*), the sample was exposed to light for 5 s using a 300-watt slide projector with a 515-nm cut-on filter (*b*). Successive spectra were then recorded after 5 (*c*), 10 (*d*), 20 (*e*), 40 (*f*), and 60 (*g*) min in the dark at 15 °C. Under identical conditions, wild-type rhodopsin was completely converted to MII, a 380-nm-absorbing species, by the time the first spectrum could be recorded after the 5-s bleach (not shown). For *B*, the E113Q, G90D double mutant (*b*) was purified from COS cells as in *A*; the absorption spectrum for the single mutant E113Q (*a*) is shown for comparison. Both spectra were recorded at pH 7.4.

to longer wavelengths. As is shown in Fig. 2B, the absorption maximum of the double mutant E113Q,G90D is at 472 nm, consistent with reprotonation of the Schiff base upon moving the counterion to position 90 in the second transmembrane segment.

Our mutagenesis studies place Gly 90 in a position oriented towards Lys 296 and at the same level as Lys 296 in the membrane, with helix II side by side with helix VII in the three-dimensional structure of rhodopsin (Fig. 3). This arrangement of the helices may hold for all G-protein-coupled receptors. It is consistent with the 9 Å projection map of rhodopsin¹⁸ and agrees with a recent model for G-protein-coupled receptors¹⁹.

We conclude that activation of opsin in the single mutant G90D results from disruption of the Glu 113–Lys 296 salt bridge, as in mutations at positions 113, 292 and 296, indicating that these mutations all activate opsin by the same molecular mechanism.

The anomalously slow bleaching of G90D (Fig. 2A) could result from an effect of the mutation on the rate of M1–M2 interconversion¹⁴. However, structural information from our double mutant experiment offers another explanation, namely that the blue-shifted intermediate formed rapidly upon exposure to light is an active, M2-like intermediate containing a protonated Schiff base (the Schiff base is not protonated in the wild-type M2 intermediate¹⁴). This presupposes that Asp 90 lies close to the Schiff base nitrogen in the active state as well as in the ground state.

Patients with stationary night blindness have severely impaired rod vision but, unlike RP patients, they show no retinal degeneration. Our results explain why very different phenotypes (night blindness and retinitis pigmentosa) can both result from essentially the same molecular defect, constitutively active opsins. The mutants associated with RP (K296E and K296M) are incapable of binding the retinal chromophore²⁰, so all the mutant protein is in the active opsin state; however, mutants associated with stationary night blindness (A292E and G90D) can bind the chromophore and are inactive in the dark. Most of the 10⁸ pigment molecules in a rod cell²¹ will therefore be tightly bound to the 11-*cis*-retinal chromophore and ready to absorb a photon of light, but as the rod cell does not contain a large excess of retinal²², at any given moment there will be a number of opsin molecules (perhaps a few hundred) with vacant binding sites. This normally presents no problem for the rod cell as wild-type opsin is inactive, but in the case of the G90D and A292E mutants these opsins are active and are present in sufficient numbers to desensitize the cell severely or perhaps even fully saturate the response (of the 10⁸ rhodopsin molecules, only ~200 need be activated to saturate the electrical response of the cell²³). According to this model, night blindness arises because the rod photoreceptor cells adapt to a dark signal coming from a relatively small number of constitutively active opsin molecules. This would account for the increased dark-adapted thresholds of G90D individuals¹². The severity of retinal disease is thus correlated with the severity of molecular defect in the opsin, with night blindness and RP representing opposite extremes of the same defect.

Constitutive activation of opsin as a cause of disease is consistent with the facts that persistent light stimulation leads to retinal degeneration in laboratory animals^{24,25}, and that a mutation in *Drosophila* arrestin, whose function is to inactivate light-activated rhodopsin, leads to retinal degeneration²⁶. We also note that mutations in two other G-protein-linked receptors have recently been reported to cause human disease as a result of constitutive activation of the receptors, namely of the thyrotropin receptor in hyperfunctioning thyroid adenomas²⁷, and of the luteinizing

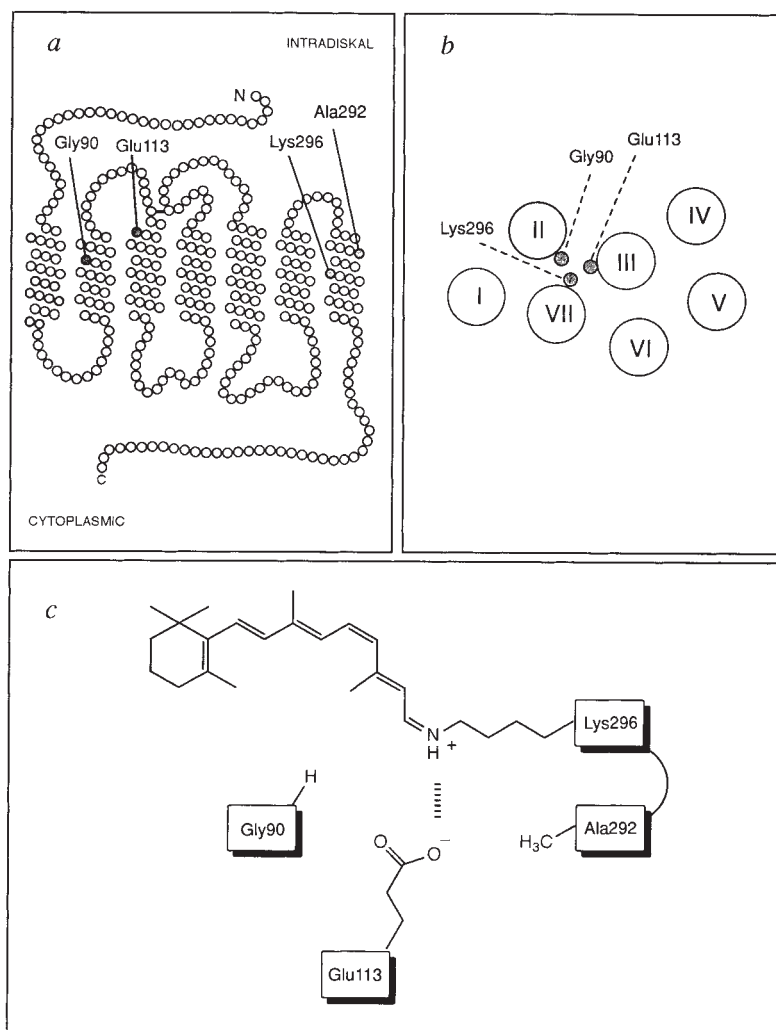


FIG. 3 Structure of rhodopsin. *a*, Schematic diagram showing the seven transmembrane α -helical segments of rhodopsin. Shaded circles indicate the four amino acid residues that constitutively activate opsin when mutated. *b*, Cross-sectional view of rhodopsin. This view is rotated 90° relative to that in *a*, looking at the helical bundle parallel to the longitudinal axes of the helices. This diagram is based on a 9 Å projection map¹⁸ obtained by cryoelectron microscopy of two-dimensional arrays of rhodopsin, and a model¹⁹ for the structure of G-protein-linked receptors. Filled circles correspond to amino acids Gly 90, Glu 113 and Lys 296. *c*, Schematic diagram of chromophore-binding site.

hormone receptor in familial male precocious puberty²⁸. Also, a dominant allele for coat pigmentation in mice encodes a constitutively active mutant of the melanocyte-stimulating hormone receptor²⁹; it is interesting that this mutation is also found in the second transmembrane segment of the receptor. □

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Cloning of a pH-sensitive K^+ channel possessing two transmembrane segments

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THE mammalian renal collecting ducts are responsible for secreting potassium ions into the urine and are a major regulatory site for potassium homeostasis¹, in which a voltage-independent pH-sensitive K^+ channel in the apical membrane plays a central role^{2,3}. Here we describe a complementary DNA encoding a novel K^+ channel from rabbit renal cortical collecting tubule cells (RACTK1). RACTK1 has the functional characteristics of the apical K^+ -permeable channel and consists of 284 amino acids, putatively with two transmembrane segments. The sequence of RACTK1, however, shows no homology to known voltage-dependent or -independent K^+ channels, and has a different K^+ -driving path and regulatory sites. The study of this protein should provide insight into K^+ homeostasis and diseases of K^+ metabolism.

Rabbit renal collecting ducts were isolated, cultured and transformed with the SV40 gene. Patch-clamping experiments revealed that cells from the third to fifth passages at confluence possessed more K^+ channels than are usually observed in growing cells. A cDNA library was constructed from the cells at confluence. A common and possibly ancestral motif of K^+ -channel sequences is an arginine repeat, Arg-X-X-Arg-X-X, in *Drosophila Shaker*. Using the sequence as a probe, plaques were hybridized at low stringency (42 °C), and colonies of 48 plaques with positive and individual complementary RNA were injected into Chinese hamster ovary cells (CHOs).

If the injected cRNA encodes the K^+ channel, the resultant resting membrane potential (E_m) of the injected CHOs should be reduced. We therefore screened for expression of the K^+ channel 48 h after the injection by measuring E_m using a current clamp with nystatin-perforated patches⁴. cRNA from one clone showed a low E_m . Measurements were made at various times after injection and E_m values (mV) were: control, -21 ± 0.9 ; 8 h, -25 ± 1.1 ; 24 h, -28 ± 3.3 ; 48 h, -52 ± 1.5 ($P < 0.01$; F -test $n = 6$); 72 h, -28 ± 4.0 ; this indicates transient expression of exogenous cRNA.

The cDNA that produced a reduction in E_m (RACTK1) was rehybridized to the library. Only one of 10^7 plaques was found to be positive. Figure 1a shows the voltage and applied current (I - V) relationship of a single cell 48 h after the injection. The water-injected control exhibited a linear I - V , suggesting a dominant chloride conductance; the cRNA-injected cells exhibited an inwardly rectified I - V relationship. Their equilibrium potentials decreased towards the K^+ equilibrium potential predicted by the Nernst equation. External Ba^{2+} (10 mM) markedly reduced the E_m (from -51 ± 2.1 to -20.8 ± 2.3 mV, $n = 6$;

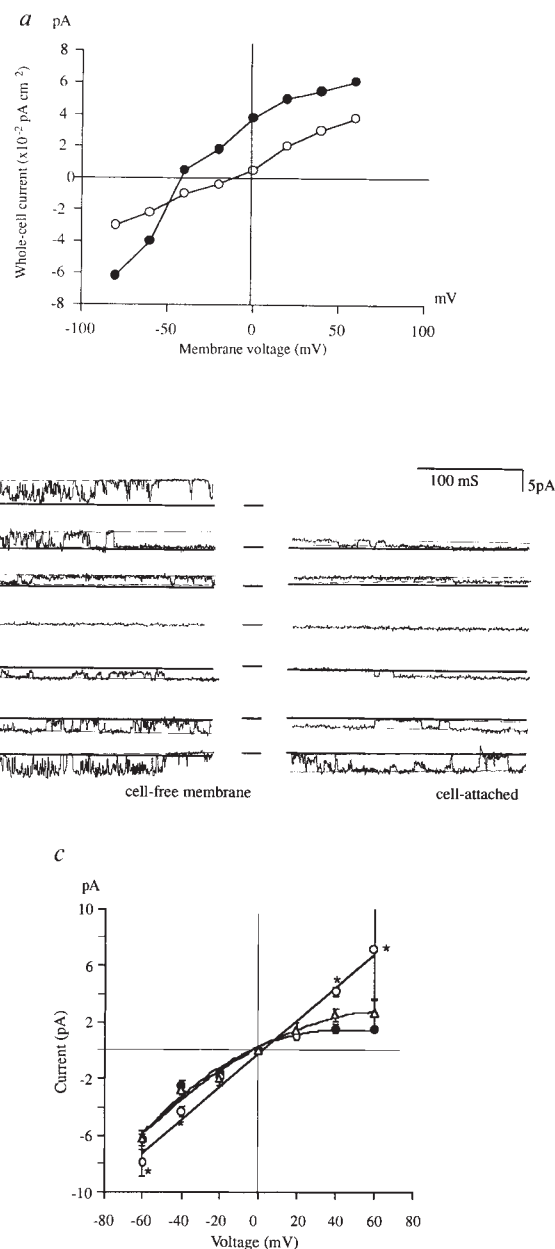
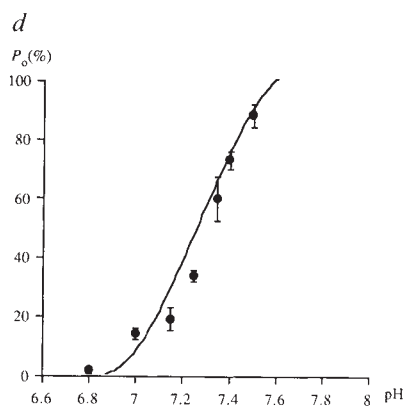


FIG. 1. Electrophysiological properties of RACTK1 channel. a, Normalized whole-cell currents ($pA\ cm^{-2}$) are plotted against membrane voltage. Each point is the mean of five measurements. ●, cRNA; ○, water. b, Traces of single-channel currents in cell-attached mode or cell-free inside-out mode. c, Current-voltage relation in cell-attached (●) and cell-free inside-out mode in the absence (○) or presence (△) of 1 mM $MgCl_2$ (mean, $n = 4$). * $P < 0.05$. d, Open probabilities (P_o) of single-channel current at a holding potential of +30 mV were plotted against bath (cytoplasmic) pH.

METHODS. Cells derived from collecting duct were maintained in hormone-enriched media. Cells at confluence were then transformed with the plasmid pSVneo3 (Bethesda Research Laboratory). Transformed

0	ATG	CCG	TTC	ATC	AGA	TCC	GAA	CCC	CGG	CTA	CGT	GTG	TTG	AGT	TGG	CAT	GCC	CGT	AAA	TAT	
0	M	P	F	I	R	S	E	P	R	L	R	V	L	S	W	H	A	R	K	Y	
60	CAA	ACT	CCA	GGC	CCG	ATG	TTC	AGA	CGG	ATG	ACG	CCC	AGA	CGA	CGG	ATG	ATT	TCC	GGG	TTA	
20	Q	T	P	P	M	F	F	R	A	M	T	P	R	R	M	I	S	G	L		
120	TTG	GAA	ATC	TCC	TGC	GGA	CGC	AAC	ACA	ATG	CGG	CTG	GCA	AGT	AAT	CCA	TAT	TGT	CGT	AAA	
40	L	E	I	S	C	G	R	N	T	M	R	L	A	S	N	P	Y	C	R	K	
180	TCT	AGC	GCA	GGG	AAT	CGG	CCG	AGA	TTG	TCA	GGC	TGG	AGG	CGC	TGG	CCC	GCT	GAT	TTG	GCC	
60	S	S	A	G	N	R	P	R	L	S	G	W	R	R	W	P	A	D	L	A	
240	GGT	TTC	AGT	AAA	TGC	ACC	ACC	GAT	TCC	TGT	AGC	ACT	TCC	GAA	TGC	ATC	ATA	AAC	GGA	GGA	
80	G	F	S	K	C	T	T	D	S	C	S	T	S	E	C	I	I	N	G	G	
300	ATT	ACC	GGG	TTT	TCC	CCC	AGA	CGC	GCC	ATT	ACC	GCA	TTA	TTG	ATA	TTG	CCC	ACG	CCA	CTT	
100	I	T	G	F	S	P	R	R	A	I	T	A	L	L	L	P	T	P	L	L	
360	TGC	AGC	GGC	AGC	AGT	GAT	TCA	GGC	GGA	ATA	CGC	CCA	TGC	GCC	ATT	TCC	CCA	CGC	CAC	TTG	
120	C	S	G	S	D	S	G	G	G	I	R	P	C	A	G	I	S	P	R	H	L
420	GCA	GGC	GGC	AGC	AGT	GAT	TCA	GGC	GGA	ATA	CGC	CCA	TGC	GCC	ATT	TCC	TGC	GAT	AAG	AAC	
140	A	A	S	S	D	S	G	G	I	R	P	C	A	G	I	S	C	D	K	N	
480	TGA	CCA	CGT	TAT	CGG	CAA	TCT	GCT	GGC	ACA	TGG	GAT	TTT	TCT	TAT	CCA	GCA	TAT	TAC	CGG	
160	V	T	T	L	S	A	I	C	W	H	M	G	F	F	L	S	S	I	L	P	
540	CGC	TCG	GGT	AAG	TTG	GTT	TCC	ACG	ACG	CGC	ACA	ATC	TTT	TTT	GGA	TCC	GAT	TTG	AAC	ATA	
180	A	S	G	K	L	V	S	T	T	A	T	I	F	F	G	S	D	L	N	I	
600	GCG	GGT	ACC	GAC	GCG	ATC	CAT	TGC	ATG	GAA	GAT	CGA	CAC	GCT	ATT	GCG	CCG	CGG	TGG	CGC	
200	A	G	T	D	A	I	H	C	M	E	D	R	H	A	I	A	P	R	W	R	
660	GCC	AGG	AAT	CAC	AAT	ATC	CGC	CAG	TTC	TGC	AAC	CGC	CGG	ATC	GTG	ATA	GTG	ATT	GAG	TCA	
220	A	R	N	H	N	I	R	Q	F	C	N	A	R	I	V	I	V	I	E	S	
720	ATG	ATC	ACT	TTC	TTC	GCC	CGC	AGT	AGC	CAG	GTC	GGC	GCA	TTA	CCG	ATC	CCG	CTG	GTT	AAC	
240	M	I	T	F	F	A	R	S	S	Q	V	G	A	L	P	I	P	L	V	N	
780	CAG	ACT	GCA	CCA	TCC	GGT	GCC	AGT	GCC	GAT	GCT	TCA	ATG	ACG	AAA	CAT	GGA	TGT	CGC	GGA	
260	Q	T	R	P	S	G	A	S	A	D	A	S	M	T	K	H	R	C	R	R	
840	AGA	AAC	CGT	AAT	TGA	CCATTTCGCC	CACCTTCGCTC	AAATCAGGTC	AACGAAACTCA	CCGGC											
280	R	N	R	N																	
900	CCCTGATTGA	TCTTTTTCAG	TAAACCGGAC	GATGTTTGTAT	ATGGGCGACG	CCAGGAACA															
960	GCATCGGCGCT	CAGAAAGTAC	ATCGTCAGCG	GCAGCAGTGA	TTTACGGCA																

FIG. 2 Nucleotide and predicted amino-acid sequences of RACKT1 channel cDNA. Nucleotides are numbered beginning with the first position of the ATG initiation codon; nonsense codons precede this ATG in one frame. The proposed transmembrane segment, M1, is underlined with dashes; M2 is doubly underlined. The less hydrophobic segment H5, between M1 and M2, is singly underlined. Possible phosphorylation sites based on consensus motifs for protein kinase (R-X-X-S/T, R-X-S, ●) and N-linked glycosylation sites (A-X-S, ○) are labelled. Both strands of the RACKT1 cDNA were sequenced using a sequencer (Applied BioChemistry).



cells were selected by incubating each dish of cells with media containing 10% FCS and 4 mg dl⁻¹ neomycin. The cells have been partly characterized previously²⁶. Poly(A)⁺ RNA (5 µg) was isolated from the cells at the fourth passage 5 days after exchange into FCS-free medium using guanidine thiocyanate extraction with ultracentrifugation in a CsCl cushion, followed by an oligo-dT column (Pharmacia) and hybridized with the probe (GGAATTCGAGTGATACGATTAGTTCGA). Positive colonies from four experiments were subcloned to pBluescript. Individual RNA was transcribed from *Apal*-digested DNA using a methylation capping analogue and T3 polymerase. Approximately 500 CHO cells were each injected with 2 ng 2 nl⁻¹ RNA. After a 2-day incubation, patch clamping was performed. Patch-clamp recordings were carried out as described previously²⁷. Records were sampled at 2,000 points s⁻¹ and analysed on a computer using Axon ver. 5.5.1 software. Open probability (P_o) was calculated as $N P_o = \sum n t_n$, where N is number of functional channels in the patch, P_o is the single-channel open probability, n represents the state of the channel (0 = closed, 1 = one open channel and so on), and t_n is the length of time spent in state n . Solution (pipette/bath): a, a filtered solution of 20 mM KCl, 130 mM potassium gluconate (K-gluconate), 3 mM HEPES, 1 mM EGTA and 100 µg ml⁻¹ nystatin⁴ (pH 7.2 using NaOH)/oxygenated Krebs-Henseleit solution (125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 1 mM NaHPO₄, 25 mM NaHCO₃ and 5 mM HEPES, pH 7.4 using NaOH); b, c, cell-free: 150 mM K-gluconate/150 mM K-gluconate (pH 7.4 using KOH) with or without 1 mM MgCl₂; d, 150 mM K-gluconate (pH 7.4)/150 mM K-gluconate (given pH).

$P < 0.01$); tetraethylammonium (TEA, 5 mM) did so partially (from -51 ± 2.1 to -36 ± 2.7 mV, $n = 6$; $P = 0.051$), but charybdotoxin (10^{-8} M) had no effect (from -53 ± 3.1 to -48 ± 3.3 mV, $n = 4$). The results suggest strongly that the conductance produced by injected cRNA was caused by the opening of K⁺ channels. Single-channel recordings in cell-attached or cell-free membranes (Fig. 1b) indicated that control CHO cells contained few endogenous K⁺ channels resembling RACKT1. The $I-V$ relation of a single channel was inwardly rectified in cell-attached membranes (Fig. 1c), linear in cell-free membranes with equivalent potassium gluconate solution and rectified when the bath contained Mg²⁺. Single-channel conductance was calculated as 78 ± 5.2 pS at 0 mV, and the open probability of the channel was not a function of voltage. Cytoplasmic Na⁺/K⁺ ions were substituted from 50/100 (mM/mM) to 75/75 or 100/50, resulting in reversal potentials from 0 to -9 ± 2.5 , -15.1 ± 3.5 or -23.4 ± 3.6 mV ($P_{K/Na} = 10$). Therefore, the cloned cDNA had encoded a voltage-independent K⁺ channel. The RACKT1-encoded K⁺ channel is sensitive to cytoplasmic pH and has a K_d (dissociation constant) at pH 7.28, similar to the intracellular pH *in vivo* (Fig. 1d).

Figure 2 shows the nucleotide sequence of RACKT1 cDNA and its deduced amino-acid sequence. Homology was sought with voltage-dependent K⁺ channels such as those of *Shaker* (refs 5, 6), *Shal* (ref. 7), *Slo* (ref. 8), *Shab* (ref. 9), mammals¹⁰, *IsK* (slow voltage-gated)¹¹ and *Arabidopsis thaliana*¹². Of these, *IsK* was the most similar, but even this was only 15% homologous in nucleotide sequence ($P < 0.36$). RACKT1 is not homologous to the voltage-independent K⁺ channel ROMK1/IRK1 (refs 13, 14), nor to the sodium channel¹⁵ (rEnaC) that is highly expressed in the kidney. Sixteen nucleotides of the probe used were matched in the second transmembrane segment, although in reverse orientation. Sequence comparison of *Shaker* S4 with the corresponding MO segment in RACKT1 showed no significant homology or amino-acid identity.

Although no homology was found, the structure of RACKT1 estimated from its hydrophobicity was very similar to that of ROMK1/IRK1 (Fig. 3a). Two putative transmembrane segments (M1 and M2) are commonly seen in both RACKT1 and ROMK1/IRK1 subunits. A less hydrophobic segment between the two transmembrane segments is also often seen (H5). Comparison of the amino-acid sequences of M1 and M2 for ROMK1/IRK1, *Slo* and *IsK* showed that they were similar (Fig.

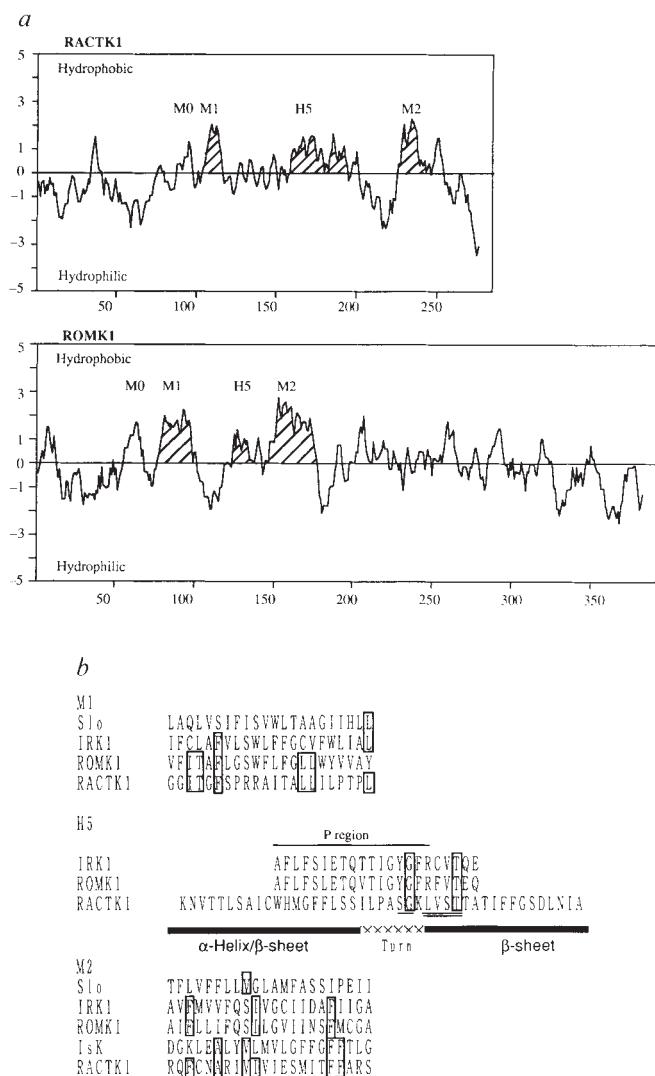


FIG. 3 Comparison of the hydrophobicity of the deduced polypeptides encoded by ROMK1 and RACTK1 cDNAs. **a**, Hydrophobicity values calculated according to Kyte and Doolittle with a window size of 10 amino acids are plotted against amino-acid position. Positive values indicate hydrophobic regions and negative values indicate hydrophilic regions. Corresponding hydrophobic peaks and H5 are hatched. **b**, Alignment of amino acids in the M1, M2 and H5 regions of K⁺ channels of ROMK1 and RACTK1 cDNA. Boxed residues are identical to those of IsK or ROMK1 at the corresponding transmembrane positions. H5: The predicted secondary structure is illustrated below. Consensus motifs of the K⁺-selective region are singly underlined and those of the TEA⁺-resistant site are doubly underlined.

3b, boxes). The H5 region of the K⁺ channels is associated with a pore-forming structure and K⁺ selectivity; the P (pore-forming) regions apparently interact to produce an ion-conducting pathway¹⁶. RACTK1 has an H5 region, and its estimated hydrophobicity suggests that it may contain a double P region (see Fig. 3a). However, the predicted secondary structure (β-turn probability value 75×10^{-6})¹⁷ shows that the whole of the P region of the K⁺ channels involving RACTK1 consists of an α-helix/β-sheet and turn followed by a β-sheet. Thus, the P region of RACTK1 was estimated to contain a serine-glycine (Fig. 3b, underlined) instead of the tyrosine-glycine couplet recently demonstrated as a requirement for K⁺ selectivity in these channels¹⁸. As serine and tyrosine are both nonpolar amino acids containing a hydroxyl group, RACTK1 K⁺ selectivity is considered to be maintained. The external surrounding 'mouth' con-

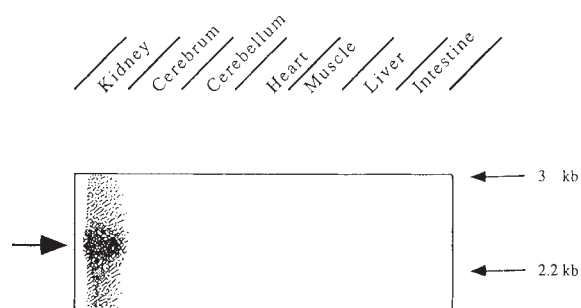


FIG. 4 Distribution of RACTK1 mRNA in tissues. Northern blot analysis of the expression of RACTK1 mRNA was performed. Lanes represent RNA from kidney, cerebrum, cerebellum, heart, skeletal muscle, liver and intestine. The migration positions of the RNA size marker are indicated on the right. A major RNA band of around 2.4 kb, which hybridizes to RACTK1 cDNA, is indicated by an arrow. The window was transferred from the original autoradiogram by image scanning.

METHODS. RNA was isolated by the guanidine thiocyanate method with organic extraction. 20 µg of RNA in each lane was transferred to a nitrocellulose membrane. A 2.2-kb *EcoRI*-*Bam*HI fragment was labelled with ³²P by random priming. Hybridization was done as described. Filters were washed with 2 × SSC, 0.1% SDS at room temperature for 20 min, then with 0.1 × SSC, 0.1% SDS at 55 °C for 20 min and autoradiographed. RNA, including a size marker, was then stained with methylene blue to confirm the transfer²⁸.

serves an aromatic residue (tyrosine or valine; Fig. 3b, double underlining), and this alignment may explain the low-affinity TEA⁺ binding¹⁹. Further, the heptad leucine repeat involved in voltage-dependent gating²⁰ is absent in RACTK1. Therefore, although RACTK1 shows little homology to previously characterized K⁺ channels, the consensus motifs of amino-acid alignment obtained by site-directed mutagenesis are consistent with physiological findings: voltage-independence, K⁺-selectivity and TEA⁺- and charybdotoxin-resistance²¹.

ROMK1/IRK1 differs from RACTK1 in amino-acid sequence in that the ATP-binding²² site in ROMK1/IRK1 cDNA is absent in RACTK1, the hydrophilic carboxy terminus is shorter and the amino terminus longer in RACTK1 than in ROMK1/IRK1 cDNA, and possible phosphorylation sites based on consensus motifs for protein kinase²³ are abundant in the N terminus of RACTK1 cDNA but in the C terminus of ROMK1/IRK1 cDNA.

Northern blot analysis of the expression of RACTK1 messenger RNA in various rabbit tissues (Fig. 4) revealed that a 2.4-kilobase (kb) mRNA for RACTK1 was detected only in the kidney.

Thus, a K⁺ channel exists in the apical membrane of the renal collecting ducts which contributes to K⁺ secretion and which is voltage-independent, pH-sensitive and inhibited by millimolar concentrations of ATP^{2,3}. RACTK1 cDNA encodes a K⁺ channel that has similar characteristics (*K_i* for ATP = 0.8 mM, *n* = 6, data not shown), and which might be a member of the ATP-inhibitable K⁺ channels widely distributed in other organs^{24,25}. □

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The sequence of the K⁺ channel described here is filed at GeneBank/EMBL/DBJ, accession number 16216. During preparation of this manuscript, a polyclonal antibody to RACK1 was prepared by M.S. This rat IgG antibody binds to the luminal side of the collecting duct of the rabbit kidney.

A cell initiating human acute myeloid leukaemia after transplantation into SCID mice

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MOST human acute myeloid leukaemia (AML) cells have limited proliferative capacity, suggesting that the leukaemic clone may be maintained by a rare population of stem cells^{1–5}. This putative leukaemic stem cell has not been characterized because the available *in vitro* assays can only detect progenitors with limited proliferative and replating potential^{4–7}. We have now identified an AML-initiating cell by transplantation into severe combined immunodeficient (SCID) mice. These cells homed to the bone marrow and proliferated extensively in response to *in vivo* cytokine treatment, resulting in a pattern of dissemination and leukaemic cell morphology similar to that seen in the original patients. Limiting dilution analysis showed that the frequency of these leukaemia-initiating cells in the peripheral blood of AML patients was one engraftment unit in 250,000 cells. We fractionated AML cells on the basis of cell-surface-marker expression and found that the leukaemia-initiating cells that could engraft SCID mice to produce large numbers of colony-forming progenitors were CD34⁺CD38[−]; however, the CD34⁺CD38⁺ and CD34[−] fractions contained no cells with these properties. This *in vivo* model replicates many aspects of human AML and defines a new leukaemia-initiating cell which is less mature than colony-forming cells.

The success in transplanting normal human haematopoietic cells^{8,9} and acute lymphoid leukaemia cells^{10,11} into immunodeficient SCID mice¹² suggested that this system might be useful for studying human myeloid leukaemias. But previous experiments indicated that primary AML cells could not be grafted into SCID mice after intravenous injection^{13,15}, and only a few samples grew locally after implantation into the peritoneum or under the renal capsule^{13,14}. As AML cells have stringent cyto-

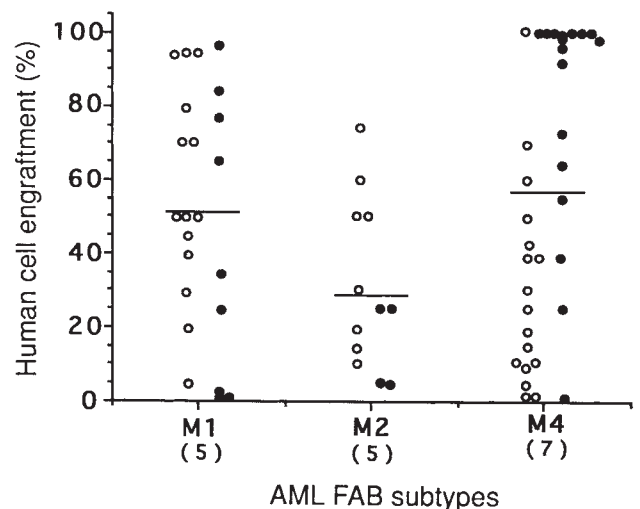


FIG. 1 Summary of human cell engraftment in the bone marrow of SCID mice transplanted with cells from AML patients, FAB subtypes M1, M2 or M4, where: M1 is AML without maturation; M2 is AML with granulocytic maturation; M4, acute myelomonocytic leukaemia; M5, acute monocytic leukaemia²². Number of donors is indicated. Engraftment was quantified by DNA analysis (open circles) or by cytology (filled circles) of Wright-stained bone marrow touch preparations 17–45 days post-transplantation. Lines represent mean level of engraftment for each FAB group.

METHODS. Bone marrow and peripheral blood were obtained after informed consent according to procedures approved by the Human Experimentation Committee. Fresh or thawed AML cells were enriched by Ficoll-density gradient centrifugation and washed in IMDM medium containing 10% FCS. For transplantation of human cells, cells (1×10^7 – 4×10^7) were injected into the tail vein of sublethally irradiated (400cGy, using a ¹³⁷Cs irradiator) SCID mice according to our established protocols⁹. PIXY321 (a fusion protein of human granulocyte-macrophage colony-stimulating factor with human IL-3) (7 µg) and human mast-cell growth factor (hMGF; *c-kit*-ligand; 10 µg) were administered on alternate days by intraperitoneal injection. Mice were bred and maintained in a defined flora colony (Ontario Cancer Institute). Grafting of human cells was quantified as described⁹. Briefly, 5 µg phenol-extracted DNA was digested with *EcoRI*, blotted onto a nylon membrane (Amersham) and probed with p17H8, a human α -satellite probe specific for human chromosome-17 sequences²³. The percentage of human cells present in the mouse tissues was estimated by comparison of the intensity of the characteristic 2.7-kb band with standard human/mouse DNA mixtures (0, 0.1, 1.0, 10 and 50% human DNA).

kin requirements *in vitro*⁷, we tested the effect of treating SCID mice, transplanted with peripheral blood cells (PBL) from AML patients newly diagnosed according to the French-American-British classification (FAB) as M1, with cytokine PIXY321 (Fig. 1 legend) and human mast-cell growth factor (MGF). DNA analysis indicated that the bone marrow from mice treated for 30–60 days contained 10–100-fold more human cells than those from untreated control mice (data not shown). We have now examined a large number of samples ($n = 17$) from patients with newly diagnosed AML of different FAB subtypes (AML M1, M2, M4) for their ability to proliferate in SCID mice. The cell source was either fresh bone marrow, fresh PBL, or banked frozen samples. All transplanted mice were treated with growth factors for the duration of the experiment (30–45 days). DNA and cytological analysis indicated that the bone marrow from 60 of 70 mice (86%) contained 10–100% human cells (Fig. 1), leading to almost complete replacement of murine haematopoiesis. Moreover, AML cells from all of the FAB subtypes (16 of 17 patients) engrafted SCID mice to high levels, indicating high reproducibility of the transplant system.

Many morphological and dissemination features characteristic of the donor's disease were reproduced in the SCID-leukaemia mice. The bone marrow of mice transplanted with AML M1 cells was extensively infiltrated with undifferentiated blast cells

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(Fig. 2a); Auer rods were also seen in some leukaemic cells (Fig. 2b). SCID mice transplanted with AML M4 cells containing an inversion of chromosome 16 had many characteristically abnormal eosinophils with large basophilic granules (Fig. 2c). Flow cytometric analysis, using CD33 and CD13, of leukaemic cells from the engrafted murine bone marrow indicated that they had an immunophenotype identical to the donor leukaemic cells (data not shown). In addition, leukaemic cells were also present in the peripheral blood of engrafted SCID mice (Fig. 2d). In contrast to mice transplanted with AML M1 and M2, some with AML M4 cells became sick or died as early as 10–20 days post-transplant, with dissemination of leukaemic blasts to the liver (Fig. 2e), lungs, spleen and kidney (data not shown). Clinically, leukaemic blasts from patients with the monocytic subtypes AML M4 and M5 disseminate more extensively to extramedullary sites than those from patients with AML M1/M2, suggesting that the SCID-leukaemia model accurately reflects biological differences between different AML subtypes.

To determine whether immature leukaemic blast colony-forming units (AML-CFU) were present in the bone marrow of highly engrafted mice, single-cell suspensions of marrow were plated in methylcellulose cultures. AML-CFU were present in mice transplanted with 11 of 11 donor samples, regardless of the FAB classification (Fig. 3a), and no progenitors of normal lineages were detected. Leukaemic cells, before and after transplantation into SCID mice, were plated at limiting dilution to compare the frequencies of AML-CFU. The assay was linear, and similar frequencies were obtained from the patient sample and the mouse bone marrow, 0.9 versus 0.3% respectively (Fig. 3b). Interestingly, the response in culture to interleukin-3 (IL-3) and human MGF of AML-CFU from the patient and the transplanted mouse was identical, indicating that neither the murine environment nor exogenous cytokine treatment selected for clones with altered responses to growth factors (Fig. 3b). Kinetic experiments were done to measure the number of human cells and AML-CFU present in the murine bone marrow over 28 days following transplantation of either 10^6 or 1.3×10^7 cells. At both cell doses, AML-CFU increased by >100-fold over 14–28 days relative to the number detected in bone marrow one day after transplantation (Fig. 3c). The total number of human cells increased by 1,000-fold over the same period (data not shown). The presence of large numbers of growth-factor-responsive AML-CFU and their extensive expansion in the bone marrow of cytokine-treated mice implied that a leukaemic stem cell more immature than AML-CFU was maintaining the progenitor pool.

Flow-sorting was used to characterize and purify the leukaemia-initiating cells. CD34 is a cell-surface marker normally expressed on a small population of bone marrow cells, including progenitor cells and pluripotent stem cells¹⁶. Expression of CD38 on CD34⁺ cells is an important marker for lineage commitment and therefore the CD34⁺CD38⁻ phenotype defines an immature human cell in normal bone marrow. Although the expression of CD34 and CD38 on AML cells is very heterogeneous¹⁷, the CD34⁺CD38⁻ phenotype is present on immature AML-CFU¹⁸. Peripheral blood cells from an AML M1 patient were separated into CD34-positive and -negative fractions and transplanted into SCID mice. Leukaemic cell proliferation and high levels of AML-CFU were observed in the bone marrow of mice transplanted with CD34⁺ cells, as in mice transplanted with unsorted populations (Fig. 3d). By contrast, four mice transplanted with CD34⁻ cells were poorly engrafted (0–0.1%) and contained no AML-CFU, except for one

mouse that had been transplanted with a high cell dose and contained only a few colonies (Fig. 3d). In two further experiments mice were transplanted with CD34⁺CD38⁻ or CD34⁺CD38⁺ cells purified from the same donor. Leukaemia cell proliferation and high levels of AML-CFU were only seen in mice transplanted with CD34⁺CD38⁻ cells (Fig. 3d). Interestingly, both populations contained comparably high numbers of

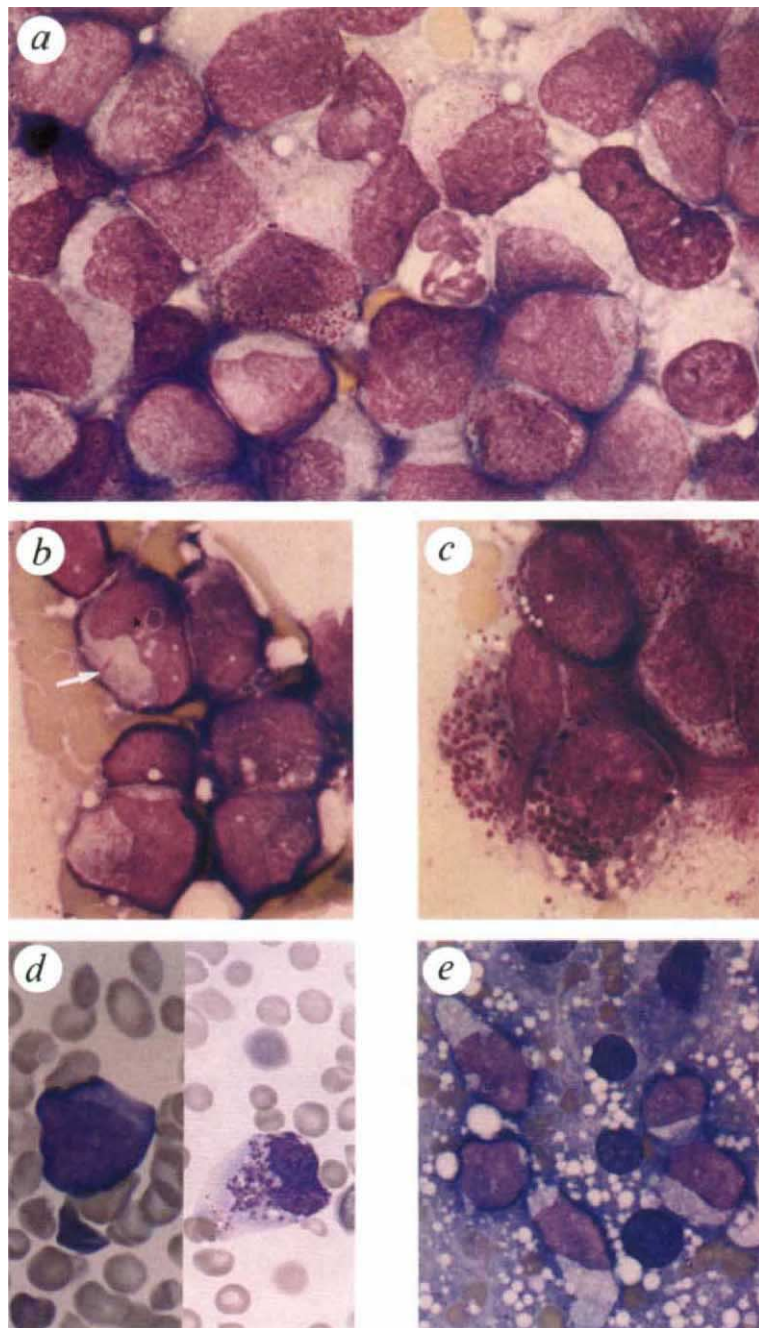


FIG. 2 Histology of cytokine-treated SCID mice injected with AML cells 3–5 weeks post-transplantation. *a*, Bone marrow touch preparation of a mouse highly infiltrated with AML M1 cells. Only one mouse neutrophil with a ring-shaped nucleus is present among the characteristic AML M1 blast cells. *b*, Mouse marrow repopulated with cells from a different AML M1 patient containing an Auer rod (arrow). *c*, Mouse marrow repopulated with cells from an AML M4 (with eosinophils and inversion of chromosome 16) patient containing characteristic eosinophils with large basophilic granules. *d*, Peripheral blood smear of a mouse repopulated with AML M4 cells. Human blast cells (left) and eosinophils (right) were present in the circulation of the mouse. *e*, Liver touch preparation of a mouse engrafted with AML M4 cells. Infiltrating leukaemic blast cells can be seen among mouse hepatocytes.

AML-CFU before injection into SCID mice, providing evidence that at least $CD34^+CD38^+$ AML-CFU could not engraft SCID mice. $CD34^+CD38^-$ cells transplanted into SCID mice did not produce any detectable normal human progenitors; all of the colony-forming cells tested (40/40) contained the same t(2;4) chromosomal translocation found in the patient's leukaemic clone.

We next investigated whether there was a linear relationship

between the number of cells injected and leukaemic engraftment, in order to develop a quantitative assay for AML-initiating cells. Peripheral blood cells from AML patients were diluted in a tenfold series from 2×10^7 cells to 2×10^4 cells before transplanting into SCID mice. In a representative experiment, DNA analysis indicated that as few as 2×10^5 cells were sufficient to initiate leukaemic proliferation (Fig. 4). Statistical analysis of the proportion of mice that had leukaemic proliferation at each cell

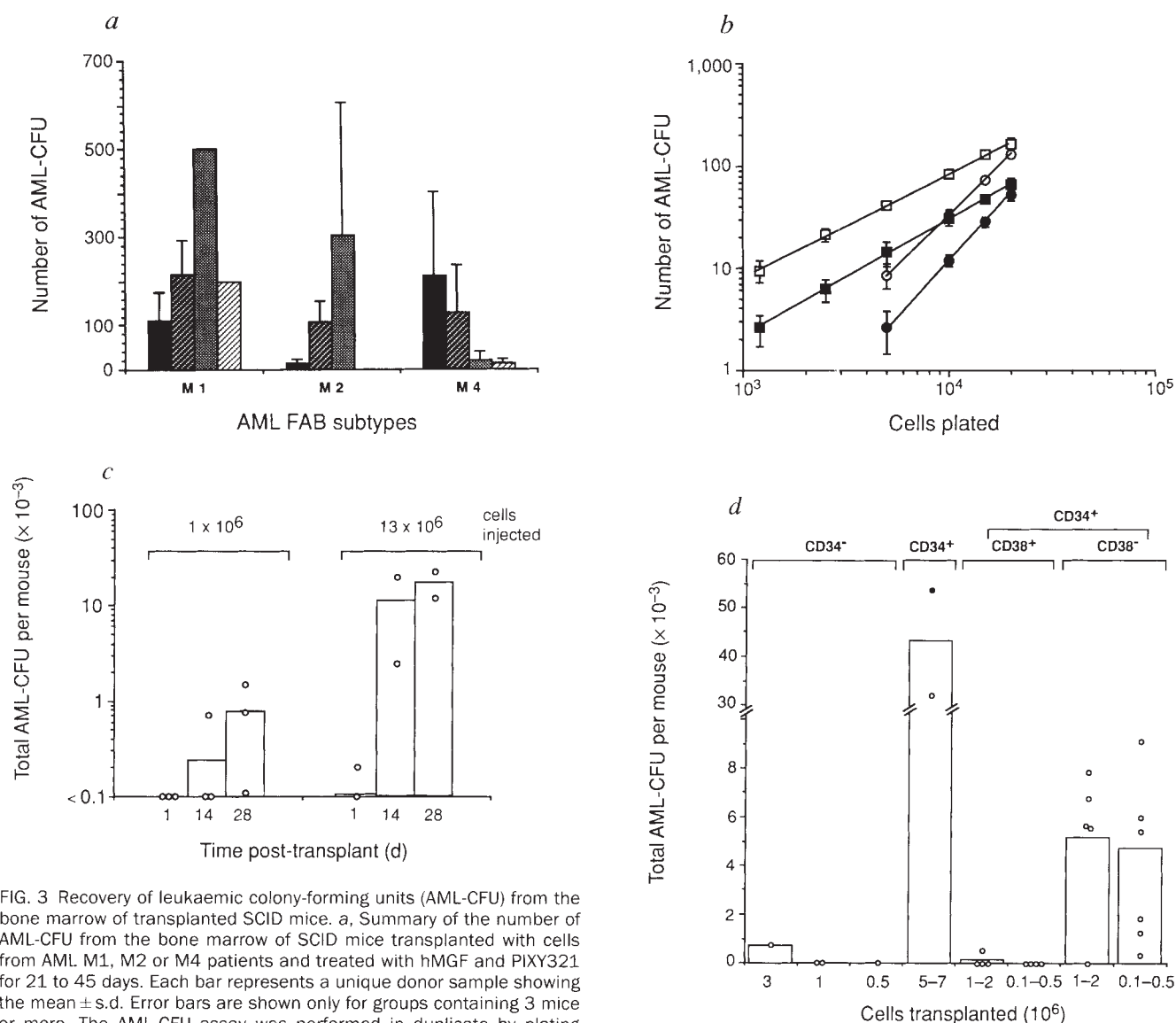
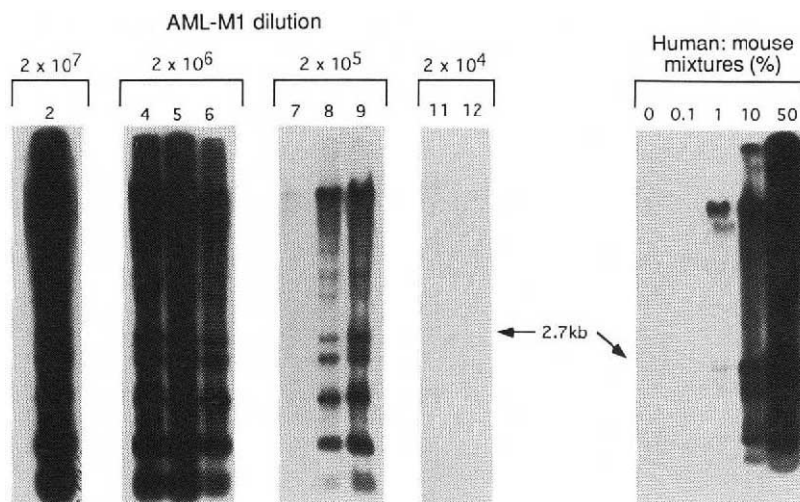


FIG. 3 Recovery of leukaemic colony-forming units (AML-CFU) from the bone marrow of transplanted SCID mice. **a**, Summary of the number of AML-CFU from the bone marrow of SCID mice transplanted with cells from AML M1, M2 or M4 patients and treated with hMGF and PIXY321 for 21 to 45 days. Each bar represents a unique donor sample showing the mean $\pm$ s.d. Error bars are shown only for groups containing 3 mice or more. The AML-CFU assay was performed in duplicate by plating 2×10^5 bone marrow cells in 0.9% methylcellulose-containing fetal bovine serum (15%), human plasma (15%), hMGF (50 ng ml⁻¹), PIXY 321 (5 ng ml⁻¹), hGM-CSF (1 U ml⁻¹), hIL-3 (10 U ml⁻¹) and human erythropoietin (2 U ml⁻¹). At day 7, leukaemic blast colonies were scored and their leukaemic identity confirmed by cytology and chromosomal analysis where applicable. No murine colonies were obtained under these selective culture conditions^{8,9}. **b**, Frequency of AML-CFU before and after transplantation. Limiting dilution analysis of AML-CFU present in the PBL of an AML M1 patient before (open symbols) and after (filled symbols) transplantation. Cell numbers indicated were plated in methylcellulose culture containing either hIL-3 (1 nM) alone (circles) or in combination with hMGF (323 pM) (squares). Bone marrow from the transplanted mouse was analysed 35 days after transplantation and treatment with hMGF and PIXY321. **c**, Expansion of AML-CFU in the bone marrow of transplanted SCID mice. SCID mice were transplanted with AML M1 cells at the cell numbers indicated and killed at 1, 14 and 28 days post-transplant. The total number of AML-CFU per mouse was determined by multiplying the AML-CFU per 2×10^5 cells plated by the total number of bone marrow cells present in the mouse. The limit

of detection was 100 AML-CFU per mouse. **d**, Measurement of AML-CFU from SCID mice transplanted with AML cells fractionated according to CD34 and CD38 expression. Three independent cell-sorting experiments were done on thawed cells containing 75% CD34⁺ cells from an AML M1 patient; of these 40% were CD38⁺. Cells were purified by fluorescence-activated cell sorting (FACStar^{PLUS}; Becton-Dickinson) or using a CD34 affinity column (Cell-Pro) (filled circle). In the first experiment, cells were separated on the basis of CD34 expression; the CD34⁺ and CD34⁻ populations were each 98% pure; in experiments 2 and 3, respectively, the CD34⁺/CD38⁺ cells were 88 and 97% pure, and the CD34⁺/CD38⁻ cells were 80 and 73% pure, being contaminated with 10 or 15% CD34⁻/CD38⁺ cells. The CD34⁺/CD38⁺ and the CD34⁺/CD38⁻ fractions from both experiments 2 and 3 contained an average of 6,525 and 8,736 AML-CFU per 2×10^5 cells, respectively. Mice were transplanted with the indicated number of purified cells and treated with cytokines. After 45 days (experiment 1) or 30 days (experiment 2 and 3), the total number of AML-CFU was determined.

FIG. 4 Determination of the frequency of the SCID leukaemia-initiating cell (SL-IC) engraftment unit by limiting dilution analysis. PBL cells from an AML M1 patient were thawed and different cell doses (2×10^4 , 2×10^5 , 2×10^6 and 2×10^7 cells) were transplanted into groups of 3 or 4 mice. Mice were treated with hMGF and PIXY321 for 1 month, after which DNA from the bone marrow was analysed for human cells as described for Fig. 1. The Southern blot is representative of four experiments with four different donors. SCID mice containing $>5\%$ leukaemic cells in the bone marrow were considered positive for the statistical analysis used to determine the frequency of SL-IC by the method of Porter and Barry²⁴. The negative mice contained from 0.1% to undetectable human cells. Ethidium bromide staining indicated that equal amounts of DNA were loaded in each gel lane.



dose, using data from four different donors transplanted into 40 mice, indicated that the frequency of the leukaemia-initiating cell in the PBL of AML-M1 patients was 1 engraftment unit per 2.5×10^5 cells (range: 1 in 1.2×10^5 to 1 in 5.3×10^5); the engraftment followed single-order kinetics as measured by the χ^2 test (95% confidence limit).

We have identified an AML-initiating cell on the basis of its ability to establish human leukaemia in SCID mice (the SCID leukaemia-initiating cell, or SL-IC). Three pieces of evidence suggest that there may be a hierarchy of leukaemic stem cells in human AML, where SL-IC are more immature than AML-CFU. First, the frequency of SL-IC in the PBL of AML M1 patients is at least 1,000-fold lower than the frequency of AML-CFU. Second, only mice transplanted with $CD34^+CD38^-$ cells developed leukaemia whereas $CD34^+CD38^+$ mice did not, despite the fact that similar numbers of AML-CFU were present in both populations before transplantation. Third, based on the low proliferative capacity of AML-CFU in liquid or long-term cultures^{4,19,20} even with maximal growth-factor stimulation, it is likely that their large expansion in SCID mice for >45 days post-transplantation is due to the proliferation and differentiation of SL-IC. In future, autologous transplantation with purged cells may address the relationship between SL-IC and the leukaemic stem cell that maintains the disease in patients. But the fact that SL-IC shares a $CD34^+CD38^-$ expression pattern similar to normal stem cells indicates that purging strategies may be difficult to develop. It will also be possible to create complementary DNA libraries²¹ from single cells to characterize genes that are expressed in SL-IC and compare them with those from normal stem cells and the more differentiated AML-CFU. Finally, a SCID-leukaemia model that reproduces many features of human AML should help us to understand the processes governing the transformation and progression of leukaemic stem cells and to test new therapeutic strategies. □

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Selectivity of MHC-encoded peptide transporters from human, mouse and rat

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MAJOR histocompatibility complex (MHC) class I molecules present peptides from degraded intracellular antigens to $CD8^+$ T cells¹. These peptides are translocated in an ATP-dependent fashion^{2–4} into the lumen of the endoplasmic reticulum (ER) for binding to class I molecules^{5,6} by means of the MHC-encoded transporters associated with antigen processing, TAP1 and TAP2. These are members of a family of proteins containing an ATP-binding cassette and form heterodimers in the ER membrane^{7–10}. Defects in the genes encoding TAP1 or TAP2 account for impaired class I assembly and antigen presentation in several human and rodent cell lines^{7,11–13}. Whereas MHC class I molecules select peptides according to binding motifs^{14–17}, it is not clear to what extent the TAP1–TAP2 transporters have peptide sequence and length specificity. Previous studies of the rat MHC class I molecule, RT1A^a, suggested a specific conveyance of peptides by rat TAP1–TAP2 (ref. 18). Here we substitute the amino- and carboxy-terminal and the penultimate amino-acid residues of model peptides to

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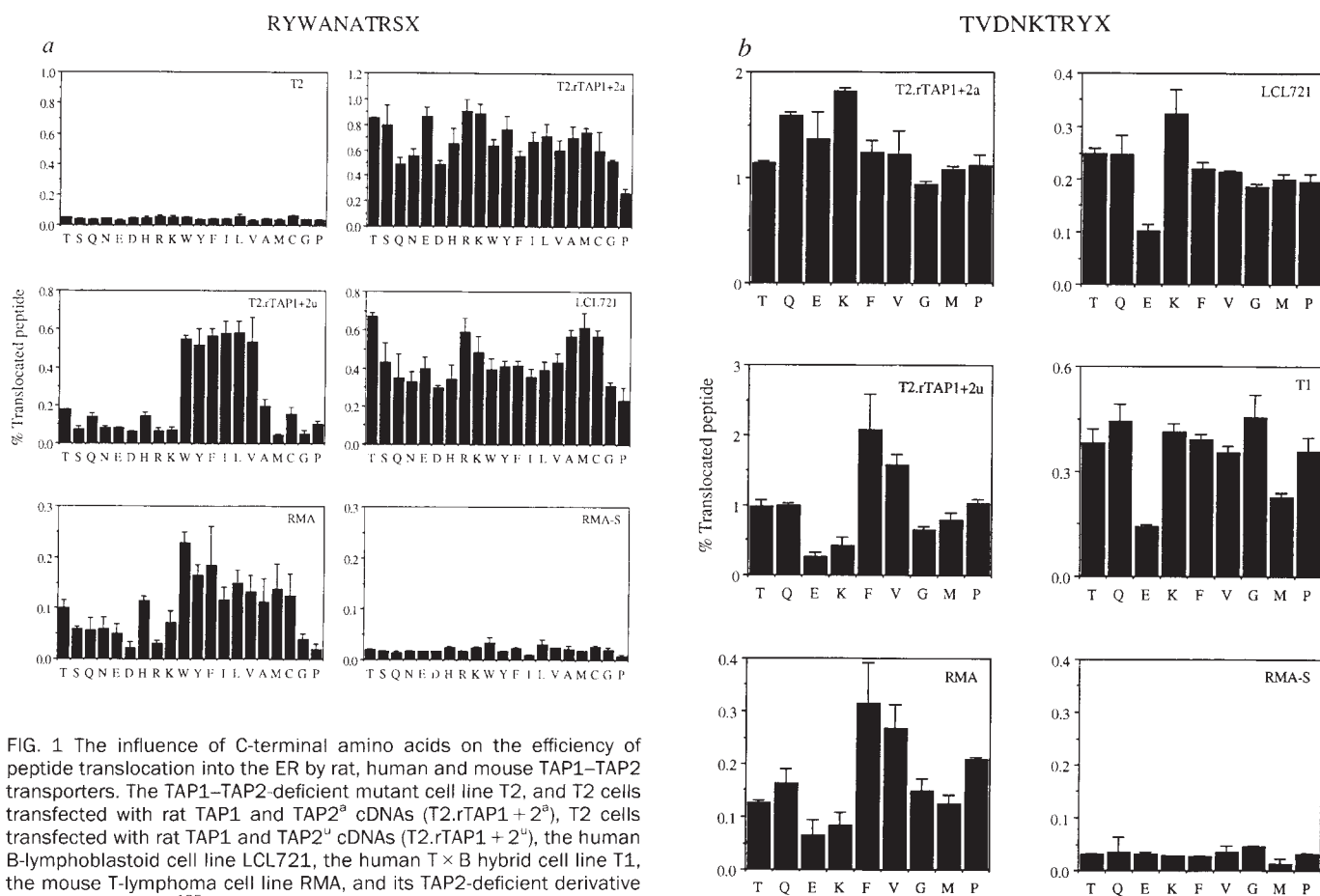


FIG. 1 The influence of C-terminal amino acids on the efficiency of peptide translocation into the ER by rat, human and mouse TAP1–TAP2 transporters. The TAP1–TAP2-deficient mutant cell line T2, and T2 cells transfected with rat TAP1 and TAP2^a cDNAs (T2.rTAP1+2^a), T2 cells transfected with rat TAP1 and TAP2^u cDNAs (T2.rTAP1+2^u), the human B-lymphoblastoid cell line LCL721, the human T × B hybrid cell line T1, the mouse T-lymphoma cell line RMA, and its TAP2-deficient derivative RMA-S were used. ¹²⁵I-labelled peptides were translocated into the ER of permeabilized cells in the presence of ATP, recovered through an N-linked glycan and quantified by γ counting. The amount of translocated peptide is expressed as percentage of the amount of input peptide. **a**, The peptide series RYWANATRSX (R..X) was used, where X is one of the 20 amino acids indicated. Peptide translocation is dependent on the expression of the two TAP subunits. The human transporter and the rat TAP1–TAP2^a transporters are fairly non-selective for the C-terminal amino acid, with the exception of Pro. The other allelic rat transporter TAP1–TAP2^u selects for peptides with C-terminal aromatic (W, Y, F) and aliphatic (L, I, V) amino acids. Mouse transporters expressed in RMA cells also show a preference for hydrophobic C-terminal residues. **b**, The peptide series TVDNKTRYX (T..X), where X is T, Q, E, K, F, V, G, M or P, was used. Rat TAP1–TAP2^a and human transporters are again rather non-selective; however, rat TAP1–TAP2^u and mouse transporters favour peptides T..F and T..V but not T..E and T..K.

METHODS. Peptides were translocated by TAP1–TAP2 essentially as described². Briefly, for every peptide translocation, 2.5×10^6 cells were

permeabilized with 2 IU ml^{-1} streptolysin O for 10 min at 37°C . Iodinated peptides ($\sim 100 \text{ ng}$, or $\sim 2 \mu\text{Ci}$) were added and incubation was continued for 20 min, then the cells were lysed. Nuclei were removed and the translocated glycosylated peptides were recovered on concanavalin A-Sepharose. After 5 washes, the associated peptides were counted. Values are means of 2–4 independent replicates $\pm$ s.e.m. R..X and T..X peptides were synthesized with a free C terminus by f-moc peptide synthesis using an ABIMED Multiple Synthesizer; peptide sequences were confirmed by mass spectrometry. The peptide R..T contained a synthesis error as reported² and was resynthesized for this study. Peptides were $>95\%$ pure by HPLC. T2.rTAP1+2^u cells were produced in the same way as T2.rTAP1+2^a cells¹⁸; T2 cells were transfected by electroporation with rat TAP1 (ref. 28) and TAP2^u (E. V. Deverson, J.C.H. and G.W.B., unpublished results) cDNAs in the pH β APr1-neo expression vector. After selection in G418 for 4 weeks, the bulk culture was sorted twice for cells expressing high levels of HLA-B5.

show that these residues influence the efficiency of transport. Human TAP and rat TAP^a translocated peptides with hydrophobic and basic C termini, whereas mouse TAP and rat TAP^u preferred peptides with hydrophobic C termini. This pattern correlates with the predominant peptide binding profiles of mouse and human class I molecules.

As the C-terminal amino acid of peptides is crucial for the binding to various MHC class I molecules¹⁵, we investigated the effect of systematic sequence variation at the C terminus of model peptides on the efficiency of TAP-dependent transport into the ER of cells permeabilized with the bacterial toxin streptolysin O (ref. 2). ¹²⁵I-labelling of tyrosine residues was used for quantitation of transported peptides, which were recovered by virtue of N-linked glycosylation in the ER. ATP-dependent peptide translocation by human TAP1–TAP2 was assessed using

the B-lymphoblastoid line LCL721 and the T × B hybrid T1 (ref. 19). The T1-derived mutant cell line T2 (ref. 19), which does not express human TAP1 and TAP2, was used as a control. T2 cells co-transfected with either rat TAP1 and rat TAP2^a (T2.rTAP1+2^a; ref. 20) or rat TAP1 and rat TAP2^u (T2.rTAP1+2^u) complementary DNAs were used to analyse the effect of the extensive allelic polymorphism in rat TAP2 on peptide transport. T2.rTAP1+2^u is a previously undescribed transfectant showing reconstituted surface expression of HLA-A2 and HLA-B5 class I molecules at levels similar to T2.rTAP1+2^a (results not shown). Furthermore, the TAP-positive mouse T-lymphoma line RMA was used with its TAP2-deficient derivative RMA-S (ref. 21). The first series of peptides had the consensus sequence RYWANATRSX (R..X), where X is one of the common 20 amino acids. As shown in Fig. 1a, the TAP-defective

cell lines T2 and RMA-S showed transport rates of <0.1% of the input peptides for all R..X peptides. In T2.rTAP1+2^a cells, the C-terminal residue of the model peptide had only a minor effect on the efficiency of translocation. Most aliphatic or aromatic amino acids, and certain charged or polar C-terminal residues, particularly favoured translocation. The peptide R..P was translocated with the lowest efficiency. Human TAP1 TAP2 expressed in LCL721 cells and T1 cells (not shown) gave rise to transport of the R..X series that was generally similar to the pattern for rat TAP1-TAP2^a, although it was generally slightly lower. The transport pattern generated by T2.rTAP1+2^u cells was quite different: only peptides having the aromatic and aliphatic residues Trp, Tyr, Phe, Ile, Leu and Val at the C terminus were efficiently transported. R..X peptides were similarly selected by the TAP1-TAP2^u-expressing rat T-cell lymphoma line C58 (ref. 22) (not shown), a pattern resembling that from mouse RMA cells. The respective transporters showed a preference for R..X peptides with hydrophobic C-terminal residues, whereas all other peptides except R..H and R..T were less efficiently translocated. Using another series of nine peptides with the consensus sequence TVDNKTRYX (T..X), we again found that T2.rTAP1+2^a and RMA cells showed low transport rates when charged Lys or Glu residues were present at the C terminus, the hydrophobic Phe and Val being preferred (Fig. 1b). Transport in T1 and LCL721 cells again was similar to that in T2.rTAP1+2^a cells, except in the case of peptide T..E, which was less efficiently transported by human TAP1-TAP2.

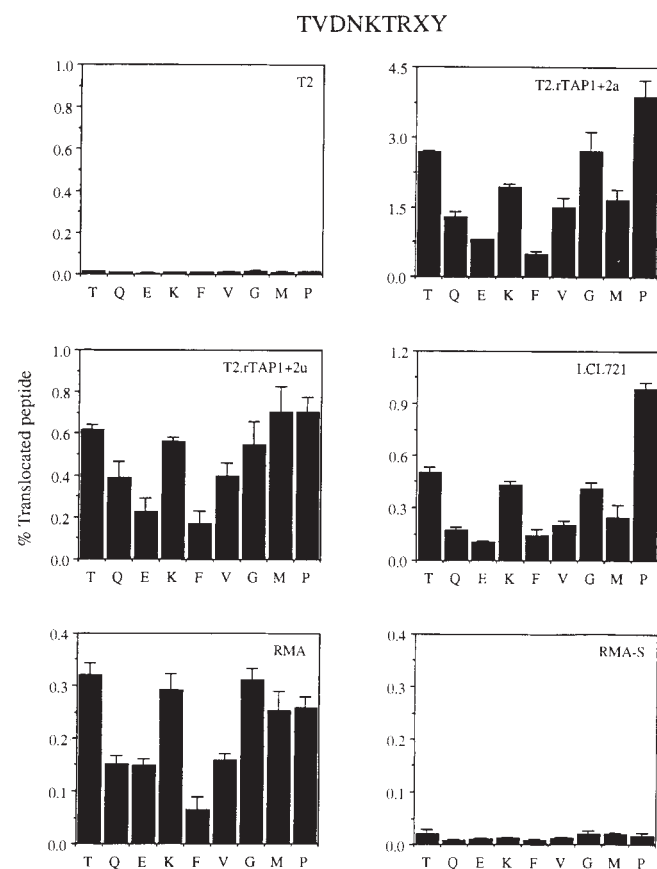


FIG. 2 The influence of the residue next to the C terminus on the efficiency of peptide transport. The peptide series TVDNKTRYX (T..XY), where X was one of the nine amino acids indicated in the legend, was iodinated and used for translocation in T2.rTAP1+2^a, T2.rTAP1+2^u, LCL721, T1 and RMA cells. T2 (not shown) and RMA-S cells were used to demonstrate TAP-dependent transport. T..FY were the worst transported peptide. Species- or allele-specific differences were minor.

The influence of the second-to-last amino acid on peptide transport was studied using nine different peptides with the consensus sequence TVDNKTRXY (Fig. 2). We found that TAP-dependent transport was influenced by the nature of the varied residue, with Phe and Glu being least preferred. There were only minor differences in transport mediated by TAPs from different species or alleles.

Next we studied the influence of N-terminal modifications on peptide transport using the peptide series XVDNKTRY (X..Y), where X was one of the common 20 amino acids (Fig. 3). The peptide VDNKTRY (without X) gave transport rates generally lower than those for X..Y peptides, indicating that the differential N-terminal amino acid was indeed influencing transport. Transport of peptides with N-terminal polar/charged and small side chains, except for P..Y, was favoured. The patterns of species- or allele-specific transport showed only minor variations.

X-ray crystallography has revealed that the peptide amino and carboxyl groups are hydrogen-bonded to conserved residues in the binding groove of class I molecules^{23,24}. We used the peptide RYWANATRSYG to assess the effect on translocation of amidation of the carboxyl group (Fig. 4a) and found that amidation gave a reduction of transport rate of ~40%, indicating that a charged C terminus facilitates transport. To determine whether a free N terminus is important for peptide transport, we methylated the N terminus of AYWANATRSYG, which gave a marked decrease in translocation efficiency but did not block it completely (Fig. 4b). As the N-formylated mitochondrial peptide MTF depends on TAP1 and TAP2 expression for presentation by M3 class I molecules¹³, a free N terminus cannot be an absolute requirement for transport. Our finding indicates that a polar N terminus may be important for interaction of the peptide with the transporter.

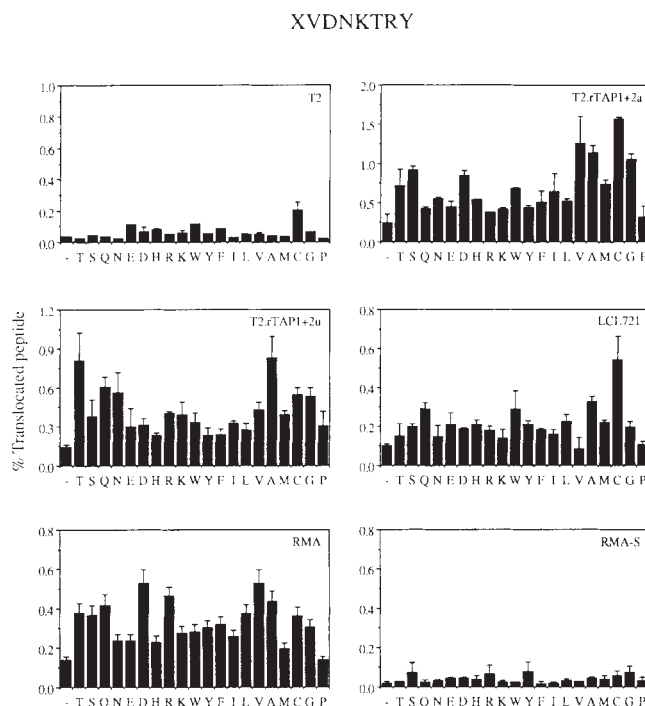


FIG. 3 The effect of N-terminal amino acids on the efficiency of peptide translocation. The peptide series XVDNKTRY (X..Y) was used, where X was one of the 20 amino acids indicated, or was omitted. Translocation of iodinated X..Y peptides was measured in T2.rTAP1+2^a, T2.rTAP1+2^u, LCL721 and RMA cells. T2 and RMA-S cells were used for control. The different N-terminal residues influenced transport rates with some preference for polar and small aliphatic amino acids. Allele- or species-specific differences were not prominent.

We conclude that the efficiency of peptide transport mediated by a given type of TAP1-TAP2 is affected by the N- and C-terminal residues and by the residue next to the C terminus. There was a marked species/allele-specific effect only in the case of the C-terminal residue. Our results with the human, mouse and rat transporters indicate that the sequence of the transporters themselves contribute to the specificity of transport. As the allelic rat transporters used here differ only with regard to their TAP2 subunit, the different transport patterns can be attributed to TAP2. The contribution of sequence variations in TAP1 from different species remains to be analysed.

Our results show that not only do MHC class I molecules select peptides according to their binding motifs, but also there is some pre-selection by the transporters. Interestingly, human transporters and certain human class I molecules with Asp residues at positions 77 and 116, such as HLA-B*2705, HLA-A11 or HLA-A*6801 (ref. 25), select for peptides with positively charged amino acids (Lys, Arg) at the C terminus^{16,17}. (Residues 77 and 116 are part of the F pocket which binds the C-terminal residue of a peptide.) In the mouse, which does not have Asp 116-containing class I sequences, such peptides are not transported efficiently, neither do they bind to class I molecules. In contrast Asp 116 is not uncommon in rat classical class I alleles²⁷. In the case of RT1A^a, which has Asp at positions 77 and 116, there is a striking preference for peptides with Arg at the C terminus in a class I-peptide binding assay (L. Young, R. Brandt, C. J. M. Melief and G. W. B., manuscript in preparation). A deficiency in the supply of such peptides by rat TAP1-TAP2^u may therefore explain the prolonged residence of RT1A^a in the ER in TAP2^u cells²², and is certainly consistent with the higher average hydrophobicity of peptides eluted from RT1A^a molecules loaded in the presence of TAP2^u compared with TAP2^a.

The similarities in peptide translocation between rat TAP1-TAP2^u and mouse TAP1-TAP2 are also consistent with the expression of the same cytotoxic T-cell target determinants (RT1A^a) when RT1A^a is expressed in mouse or rat TAP2^u cells. RT1A^a is expressed exclusively in the A^a form in L cells²² and in RMA cells (L. Young, unpublished results). It remains to be seen how much the preference of the transporters for certain peptides observed here *in vitro* reflects the transport of intracellular peptides in intact cells, which might eventually supply class I molecules with less efficiently transported peptides in amounts sufficient for antigen recognition. □

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Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1

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INTRACELLULAR signalling following mitogenic stimulation of quiescent cells involves the initiation of a phosphorylation cascade that leads to the rapid and reversible activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (refs 1, 2). MAP kinase activation is mediated by dual phosphorylation within the motif Thr-Glu-Tyr by MAP kinase kinase (MEK)³. Following activation, the MAP kinases translocate into the nucleus where they phosphorylate several transduction targets, including

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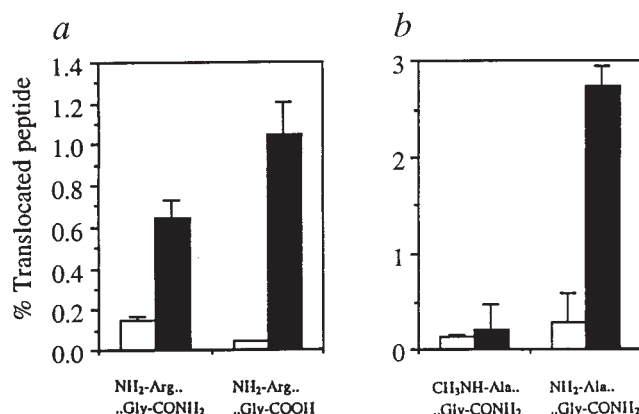


FIG. 4 a, Blocking of the C terminus decreases translocation. The peptides RYWANATRSRG having either a free C terminus (NH₂-Arg-..Gly-COOH) or a C terminus with an amide group (NH₂-Arg-..Gly-CONH₂) were iodinated and a similar amount of radioactive input peptide was translocated in permeabilized T2 cells (open bars) and T2.rTAP1 + 2^a cells (filled bars) for 20 min. The translocated peptide is expressed as percentage of the input. Amidation reduces the translocation rate by ~40%. b, A free N-terminus is essential for efficient translocation of a model peptide. The peptide AYWANATRSRG methylated at its N terminus (CH₃NH₂-Ala-..Gly-CONH₂) and its non-methylated counterpart (NH₂-Ala-..Gly-CONH₂) were used in a. Methylation of the N terminus drastically decreases the efficiency of peptide translocation.

METHODS. Translocation and iodination are described in Fig. 1 legend. The peptides Arg-..Gly and Ala-..Gly were synthesized either as free acids or acid amides by f-moc synthesis. The identity of the peptides was confirmed by mass spectrometry. The N-terminus of the peptide Ala-..Gly-CONH₂ was methylated by reductive methylation of the t-boc deprotected peptide coupled to the resin. Peptide (260 mg) and resin were incubated at room temperature for 48 h with 1 ml acetic anhydride in 5 ml pyridine. The protective groups on the amino-acid side chains were then removed and the peptide released from the resin. Quantitative methylation was confirmed by HPLC.

transcription factors⁴⁻⁷. We have previously identified PAC1 as an immediate-early mitogen-inducible tyrosine phosphatase in nuclei of T cells⁸. Here we present several lines of evidence indicating that PAC1 is a physiologically relevant MAP kinase phosphatase. Recombinant PAC1 *in vitro* is a dual-specific Thr/Tyr phosphatase with stringent substrate specificity for MAP kinase. Constitutive expression of PAC1 *in vivo* leads to inhibition of MAP kinase activity normally stimulated by epidermal growth factor, phorbol myristyl acetate, or T-cell receptor crosslinking. The inactivation of MAP kinase by PAC1 results in inhibition of MAP kinase-regulated reporter gene expression.

The catalytic domain of PAC1 (amino acids 127-314; cPAC1) was expressed as a soluble glutathione *S*-transferase (GST) fusion protein, and the enzymatic activity of the highly purified GST-cPAC1 was assayed using a variety of substrates. In a screen of potential physiological substrates, we used recombinant ERK2 that had been phosphorylated by activated MEK using [γ -³²P]ATP as substrate for the phosphoryl transfer. ERK2 was dephosphorylated *in vitro* by PAC1 at concentrations as low as 2 μ M and did not occur with tenfold more catalytically inactive PAC1 in which Cys 257 had been replaced with serine (Fig. 1a). MEK activates ERK2 as a result of phosphorylation on Thr 183 and Tyr 185 (ref. 9). In some preparations of the GST-ERK2 substrate, we and others have observed phosphorylation on tyrosine and serine that was catalysed in the purified ERK2 preparation alone (Fig. 1, and ref. 10), although such phosphorylation does not activate ERK2 kinase activity. Phosphoamino acid analysis of PAC1-treated ERK2 demonstrated a loss of phosphate from threonine and tyrosine, whereas the non-MEK-catalysed phosphoserine was unaffected (Fig. 1a). A time course of ERK2 dephosphorylation revealed that the tyrosine residue was dephosphorylated completely before threonine. However, vanadate inhibited removal of phosphate from either amino acid (Fig. 1a). As expected, PAC1 treatment of activated ERK2 resulted in an inhibition of kinase activity towards myelin basic protein (MBP) (Fig. 1b). The reversibility of PAC1-mediated dephosphorylation was shown by the reactivation of dephosphorylated ERK2 activity following MEK treatment in the presence of vanadate (Fig. 1b). PAC1 phosphatase activity appears to be remarkably specific, because several other tyrosine-phosphorylated (RAYTIDE (a protein-tyrosine kinase substrate; Oncogene Science), angiotensin, casein, v-Abl) or serine/threonine-phosphorylated (casein or MEK) substrates were not dephosphorylated *in vitro* by recombinant PAC1 (Y.W. and K.K., unpublished results).

To test directly whether ERK2 is a substrate for PAC1 *in vivo*, we used a transient co-transfection assay with plasmids encoding ERK2 and either wild-type or the Ser-257 mutant of PAC1 (PAC1-S257). The levels of ERK2, PAC1 and the PAC1-S257 proteins detected in lysates from transfected cells are shown (Fig. 2a). Tyrosine phosphorylation of ERK2 was dependent upon epidermal growth factor (EGF)- or phorbol ester (PMA)-mediated signals (Fig. 2b). In activated cells, the constitutive presence of wild-type PAC1 but not of PAC1-S257 suppressed the level of tyrosine phosphorylation of ERK2 to the background level of non-transfected cells (Fig. 2b). In parallel with the PAC1-mediated loss of phosphotyrosine on activated ERK2, a significant loss of ERK2-catalysed MBP phosphorylation was observed (Fig. 2c). The MBP kinase activity assay has a lower threshold of detection for activated MAP kinase as compared to the level detectable by anti-phosphotyrosine antibodies. Extensive PAC1-catalysed dephosphorylation of ERK2 did not occur following mixing and incubation of lysates from COS 7 cells separately transfected with ERK2 and PAC1 (not shown). Also, lysates from PAC1 and PAC1-S257-transfected cells had equivalent MEK activity as assayed by an immune complex kinase assay *in vitro* (not shown), suggesting that PAC1 did not inhibit signalling events upstream of ERK2 phosphorylation.

To analyse the consequences of expressing PAC1 constitutively at moderate levels, we established permanently transfected

Jurkat T-cell clones. The kinetics of ERK2 activation were investigated relative to the levels of PAC1 in parental Jurkat cells and in the PAC1-transfected Jurkat clone, G6 (Fig. 3). In unstimulated cells, the 32K PAC1 protein was not detectable in Jurkat, but a low steady-state level of PAC1 was observed in G6 cells (Fig. 3a). Following stimulation of Jurkat, PAC1 was observed as early as 30 min and continued to increase up to 60 min, whereas maximal levels of PAC1 were reached in G6 by 30 min after stimulation. In addition to PAC1, we observed a crossreactive, mitogen-inducible 40K protein (indicated by an asterisk in Fig. 3a) in Jurkat and G6 that was not recognized by antibodies to CL100, a previously described 39K protein phosphatase¹¹. ERK2 activation was followed by determining kinase activity toward MBP at various times after stimulating

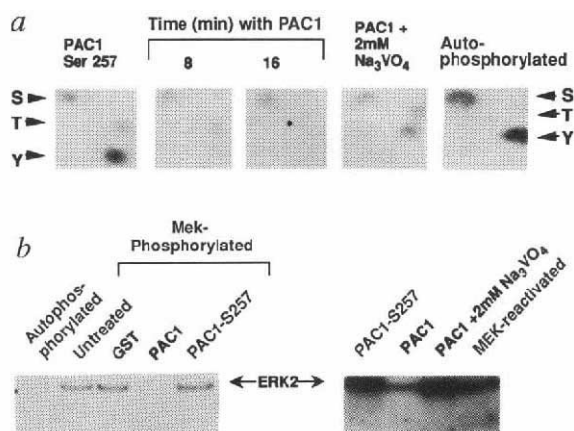


FIG. 1 PAC1 is a dual-specific Thr/Tyr phosphatase for ERK2. a, Phosphoamino acid analysis of autophosphorylated ERK2 and ³²P-GST-ERK2 incubated with GST-cPAC1 (hPAC amino acids 127-314) in the absence or presence of vanadate (2 mM) or with GST-cPAC1-S257. S, serine; T, threonine; Y, tyrosine. b, *In situ* protein kinase activity was determined for MEK-activated GST-ERK2 that was untreated, treated with GST-cPAC1 ($\pm$ 2 mM vanadate), or with GST-cPAC1-S257, or reactivated with immunoprecipitated MEK.

METHODS. a, After serum starvation and stimulation with phorbol myristyl acetate (PMA), confluent CV-1 cells were lysed in 25 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 25 mM β -glycerophosphate, 1 mM PMSF and 10 μ g ml⁻¹ leupeptin. Cell extracts were incubated for 2 h with protein A-agarose (Gibco-BRL) which had been prebound to antibody against the 16 N-terminal amino acids of MEK. GST-ERK2 (1 μ g) activation was carried out by incubating for 10 min with immunoprecipitated MEK in 50 μ l kinase buffer (25 mM HEPES, pH 7.4, 10 mM MgCl₂, 50 μ M [γ -³²P]ATP (10 μ Ci nmol⁻¹)). Phosphorylated MAP kinase (0.06 μ M) was incubated with either 20 μ M GST-cPAC1-S257 for 16 min or 20 μ M GST-cPAC1 for various time intervals in the absence of phosphatase inhibitor or for 10 min in the presence of 2 mM sodium vanadate. Dephosphorylation reactions were done in 25 μ l 50 mM HEPES, pH 7.5, and 5 mM dithiothreitol at 30 °C. Although complete dephosphorylation of GST-ERK2 occurred in 1 h with GST-PAC1 concentrations as low as 2 μ M, higher levels were used for shorter time intervals. Proteins were resolved by PAGE²⁰, electroblotted onto PVDF membrane and analysed by autoradiography. Phosphorylated amino acids were identified by phosphoamino acid analysis using two-dimensional electrophoresis on thin-layer chromatography plates²¹. Autophosphorylated MAP kinase was prepared by incubating recombinant GST-ERK2 (1 μ g) in 50 μ l kinase buffer for 1 h. b, Autophosphorylation and MEK-activation of GST-ERK2 were done as already described, except that 50 μ M ATP was used. MEK-phosphorylated GST-ERK2 (0.06 μ M) was untreated or incubated for 30 min with 20 μ M GST alone, GST-cPAC1 or GST-cPAC1-S257. Autophosphorylated GST-ERK2 was used as a negative control for MBP phosphorylating activity (left). MEK-activated MAP kinase was incubated with GST-cPAC1-S257 or with GST-cPAC1 ($\pm$ 2 mM vanadate) for 10 min. Following dephosphorylation in the absence of vanadate, GST-ERK2 was reactivated in the presence of 2 mM vanadate by immunoprecipitated MEK (right). After PAGE, *in situ* MAP kinase activity for MBP was determined²².

Jurkat and G6 cells (Fig. 3b). After mitogenic stimulation of Jurkat, ERK2 kinase activity was observed within 5 min and decreased between 30 and 60 min, coinciding with the increased expression of PAC1. By contrast, G6 cells demonstrated transient ERK2 kinase activity that was detected at 5 min but not at 30 or 60 min. The level of ERK2 activity in G6 cells at 5 min

after stimulation was ~20–40% the level in comparable Jurkat cells. Relative to Jurkat cells, G6 showed dramatically reduced amounts of phosphotyrosine on ERK2 following stimulation, although approximately equivalent amounts of ERK2 were present in both cell lines (not shown). These results demonstrated that the loss of activated ERK2 correlates with the accumula-

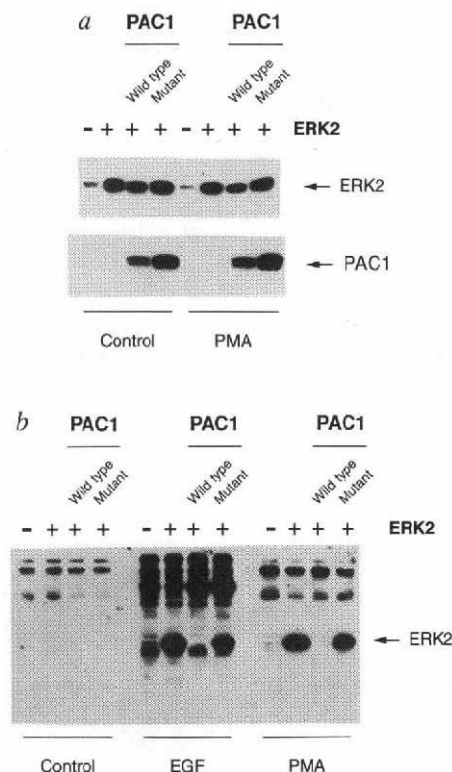
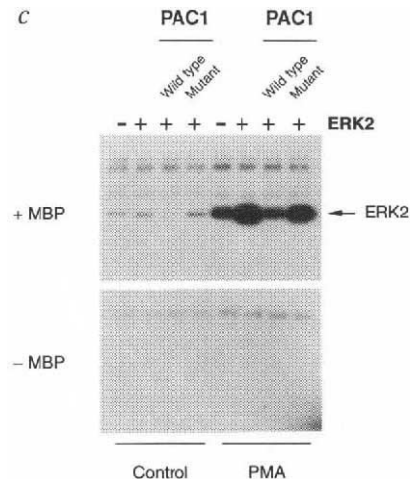


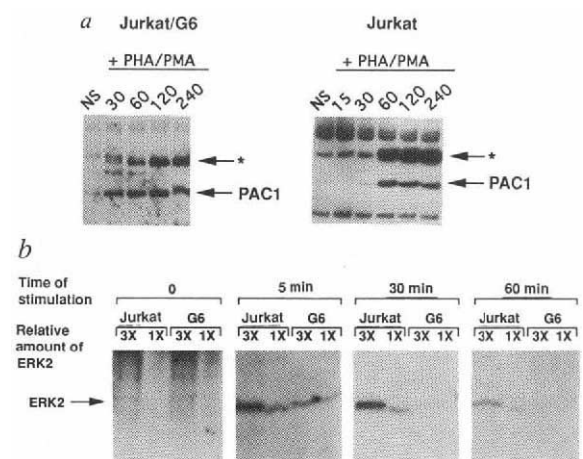
FIG. 2 MAP kinase activation is suppressed by expression of the PAC1 phosphatase. Catalytically active and inactive forms of PAC1 were co-expressed with the MAP kinase ERK2 in COS-7 cells. Cells were untreated or treated with 5 nM EGF or 10 nM PMA for 5 minutes before collecting. *a*, The level of ERK2 and PAC1 expression in untreated and PMA-treated COS cells was investigated by western blot analysis. *b*,



Tyrosine phosphorylation in extracts from untreated, EGF- or PMA-treated COS cells was examined by western blotting. *c*, Map kinase activity in extracts from untreated and PMA-treated COS cells was measured using an *in situ* renaturation assay with the substrate myelin basic protein (MBP).

METHODS. Transient transfections of COS-7 cells were done as described²³. Cells were co-transfected with 2 µg of the human ERK2 expression plasmid (pCMV-p41^{mapk}) and 2 µg of a PAC1 expression plasmid (PMT2T-PAC1 or PMT2T-PAC1-S257)⁸. Total DNA in the transfection was maintained at 10 µg using pUC13 as carrier DNA. Cells were collected 48 h after transfection. Western blot analysis was done using anti-MAP kinase monoclonal antibody 107 (R.J.D., unpublished results) and the anti-phosphotyrosine antibody PY20 (ICN) with enhanced chemiluminescence detection (Amersham). MAP kinase activity was detected after polyacrylamide gel electrophoresis using an *in situ* protein kinase assay in the absence and presence of the substrate MBP²⁴.

FIG. 3 Endogenous MAP kinase inactivation correlates with PAC1 expression in Jurkat T cells. Jurkat cells or a clone of Jurkat (Jurkat/G6) permanently transfected with an expression vector containing a spleen focus-forming virus LTR regulating the expression of a human PAC1 cDNA, were stimulated for various times with 1 µg ml⁻¹ phytohaemagglutinin (PHA) and 10 ng ml⁻¹ PMA. Cellular extracts were analysed for PAC1 expression and ERK2 activation. *a*, Steady-state PAC1 levels were measured by western blot analysis of immunoprecipitated PAC1. Stimulation times (in min) are shown above each lane. NS, not stimulated. Asterisk arrows show the 40K band discussed in the text. *b*, MAP kinase activity of immunoprecipitated ERK2 from cellular extracts was measured using an *in situ* assay with MBP as substrate. METHODS. *a*, At various times after activation, Jurkat or G6 cells (5 × 10⁶) were lysed with RIPA buffer²⁵. Extracts were incubated overnight with three monoclonal antibodies against PAC1: P9D10, P12B11 and P10C5 (P.N.J. and K.K., unpublished) and protein G-Sepharose (Gibco-BRL). Washed immunoprecipitates were subjected to SDS-PAGE and western blot analysis with rabbit anti-PAC1 peptide (279–291) antibody⁸ and ECL (Amersham). Bands above and below the arrows are crossreactions to murine IgG. *b*, At various times after activation, Jurkat or G6 cells were lysed with RIPA buffer²⁵. Protein (500 µg) from each sample was denatured in 0.5% SDS for 3 min at 90 °C, diluted to 0.1% SDS with RIPA buffer, and immunoprecipitated with monoclonal anti-MAP kinase antibody (2033) coupled to agarose (Zymed). Washed



immunoprecipitates were eluted in SDS sample buffer and divided into four. To test for linearity in the assay, 3 parts and 1 part from each sample were separately resolved on SDS-PAGE containing MBP. *In situ* kinase activity was determined as described²².

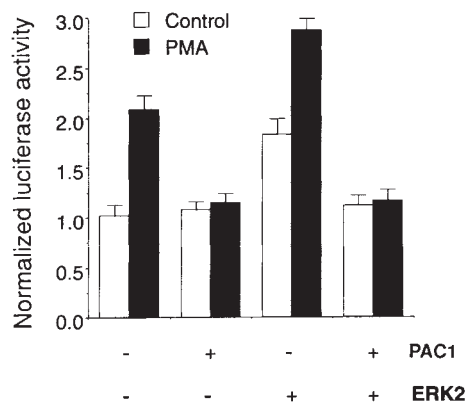


FIG. 4 PAC1 inhibits a MAP kinase signal transduction pathway leading to increased gene expression. The effect of PAC1 on the SRE-mediated expression of a luciferase reporter gene was examined using a transient transfection assay. The reporter plasmid pfos-Tk-Luc contains c-fos enhancer sequences including the SRE element (nucleotides -357 to -276) cloned upstream of a fragment of the thymidine kinase promoter (nucleotides -200 to 70) and the firefly luciferase gene.

METHODS. Transient transfections of CV-1 cells were done as described²³. Cells were transfected with 2 µg of a luciferase reporter plasmid (pfos-Tk-Luc), 1 µg of a control plasmid (pCH110) expressing β-galactosidase activity, 1 µg of the ERK2 expression plasmid pCMV-p41^{mapk}, and 1 µg of the PAC1 expression plasmid pMT2-PAC1 (ref. 8). Total DNA in the transfection was maintained at 10 µg using pUC13 as carrier DNA. Some cells (black bars) were stimulated with 10 ng ml⁻¹ PMA. The cells were collected 48 h after transfection and the extracts obtained were assayed for β-galactosidase and luciferase activity²³. The data are presented as the activity ratio of luciferase (light units per mg protein) and β-galactosidase (absorbance units per min per mg protein) (mean ± s.d., n=3).

tion of endogenous PAC1 protein in Jurkat, and that ERK2 phosphorylation and activity in G6 cells are modulated by levels of PAC1 that are substantially lower than those of transiently transfected COS cells.

PAC1 is a nuclear phosphatase that is likely to act upon MAP kinases that are translocated into the nucleus following activation⁶⁻⁸. To examine the effect of PAC1 expression on a nuclear event linked *in vivo* to MAP kinase activation, we used a transient transfection assay that measures the transcriptional activity of the c-Fos serum response element (SRE). In transiently transfected CV-1 cells, SRE-regulated reporter gene expression increased ~2-fold following PMA treatment. This PMA-stimulated expression was eliminated in the presence of constitutive PAC1 (Fig. 4). Transfection with ERK2 increased the basal SRE-mediated reporter gene expression, and this was augmented by PMA treatment. PAC1 coexpression eliminated the increased basal and PMA-stimulated SRE activity resulting from ERK2 expression. These results show that increases in SRE activity resulting from PMA treatment or from increased ERK2 expression and activation are inhibited *in vivo* by PAC1. Although the specific nuclear events that act upon the SRE are not directly addressed by these experiments, a PMA-stimulated, MAP kinase-mediated pathway involving the SRF/Elk-1 ternary complex has been described^{12,15}.

A mitogen-induced phosphatase in fibroblasts, 3CH134/CL100, has been described^{11,16}. 3CH134 has ~80% similarity with the carboxy-terminal 15K catalytic domain of PAC1, whereas the amino-terminal region of each protein (corresponding to potential regulatory domains) share ~30% similarity^{11,16}. PAC1 is maximally expressed in haematopoietic tissues, whereas 3CH134 is expressed predominantly in several other tissues^{8,16}. 3CH134 has been shown to dephosphorylate ERK1 and ERK2 *in vitro*^{10,17,18} and to dephosphorylate ERK2 in transiently transfected COS cells¹⁹. The presence of more than one ERK phos-

phatase that can be distinctly regulated represents a means by which pleiotropic kinase activation could be differentially modulated. □

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Evolutionary conservation of components of the protein translocation complex

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PROTEIN translocation into the mammalian endoplasmic reticulum requires the Sec61p complex, which consists of three membrane proteins¹. The α-subunit, the homologue of Sec61p of yeast²⁻⁴, shows some similarity to SecYp⁵, a key component of the protein export apparatus of bacteria^{6,7}. In *Escherichia coli*, SecYp is also associated with two other proteins (SecEp and band-1 protein)^{8,9}. We have now determined the sequences of the β- and γ-subunits of the mammalian Sec61p complex. Sec61-γ is homologous to SSS1p, a suppressor of sec61 mutants in *Saccharomyces cerevisiae*, and can functionally replace it in yeast cells. Moreover, Sec61-γ and SSS1p are structurally related to SecEp of *E. coli* and to putative homologues in various other bacteria. At least two subunits of the Sec61/SecYp complex therefore seem to be key components of the protein translocation apparatus in all classes of organisms.

The Sec61p complex was purified from canine pancreatic microsomes¹. Partial amino-acid sequences were determined for

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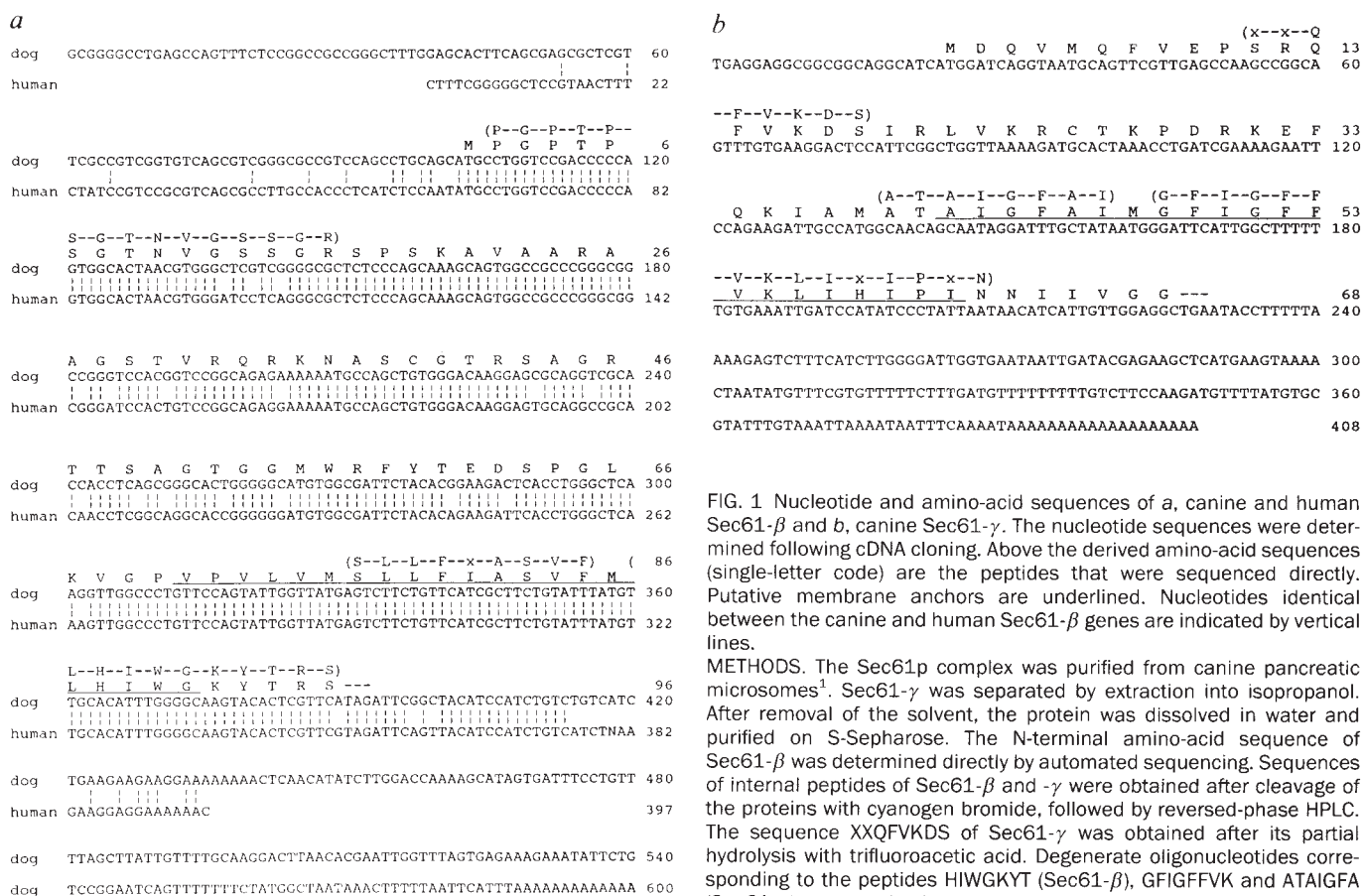


FIG. 1 Nucleotide and amino-acid sequences of *a*, canine and human Sec61- β and *b*, canine Sec61- γ . The nucleotide sequences were determined following cDNA cloning. Above the derived amino-acid sequences (single-letter code) are the peptides that were sequenced directly. Putative membrane anchors are underlined. Nucleotides identical between the canine and human Sec61- β genes are indicated by vertical lines.

METHODS. The Sec61p complex was purified from canine pancreatic microsomes¹. Sec61- γ was separated by extraction into isopropanol. After removal of the solvent, the protein was dissolved in water and purified on S-Sepharose. The N-terminal amino-acid sequence of Sec61- β was determined directly by automated sequencing. Sequences of internal peptides of Sec61- β and - γ were obtained after cleavage of the proteins with cyanogen bromide, followed by reversed-phase HPLC. The sequence XXQFVKDS of Sec61- γ was obtained after its partial hydrolysis with trifluoroacetic acid. Degenerate oligonucleotides corresponding to the peptides HIWGYK (Sec61- β), GFIFGVK and ATAIGFA (Sec61- γ) were synthesized, labelled and used to screen a cDNA library derived from MDCK cells. Human Sec61- γ was cloned from a cDNA library from HeLa cells, using as a probe the canine cDNA clone labelled by random priming. All clones were sequenced in both strands. Sequences are deposited in the EMBL database under accession numbers: canine Sec61- β , L25052; human Sec61- β , L25085; canine Sec61- γ , L25086.

the β - and γ -subunits and the corresponding complementary DNAs were cloned. The cDNAs of canine and human Sec61- β and of canine Sec61- γ code for amino-acid sequences that contain the expected peptides (Fig. 1*a*, *b*). The predicted sizes of the proteins (M_r s 10,000 and 7,500) agree reasonably well with those determined by SDS-PAGE electrophoresis (14,000 and 8,000 M_r , respectively)¹. Both proteins are predicted to span the membrane once, with a hydrophobic segment close to the C terminus, and have their N terminus located in the cytoplasm. They seem to belong to a class of 'tail-anchored' membrane proteins¹⁰ which includes the v- and t-SNAREs implicated in vesicular transport¹¹.

A homologue of the mammalian Sec61- β was found in the GenBank database in the plant *Arabidopsis thaliana* (35% identical amino acids; Fig. 2*a*). Sec61- γ is similar to a protein of rice (72% identical amino acids; Fig. 2*b*) and to partial sequences of cDNAs of mice and *Caenorhabditis elegans* (data not shown). Of particular interest is the identity of 45% amino acids between Sec61- γ and SSS1p (Fig. 2*b*), a suppressor of *sec61* temperature-sensitive (ts) mutants in *S. cerevisiae* (ref. 12, and T.S. and S.J., unpublished results) which is essential for protein translocation and for the viability of yeast cells¹². Thus, yeast also seems to have a Sec61p complex that includes homologues to the mammalian Sec61- α and Sec61- γ .

Homologues of Sec61- γ were also found in prokaryotes. A sequence in the archaeobacterium *Sulfolobus solfataricus* contains 20% identical amino acids (Fig. 2*b*), and all Sec61- γ sequences are related to SecEp of *E. coli* and SecEp-like sequences of various other bacteria (Fig. 2*c*). The homologies among the

members of the Sec61- γ /SSS1/SecEp family are statistically significant as analysed with a multiple alignment computer program (Fig. 2 legend). The gene for the Sec61- γ homologue of *S. solfataricus* is found in a similar arrangement on the genome as the *secE* genes of eubacteria, preceding sequences homologous to the transcription factor nusG and to the ribosomal proteins L11 and L1 of *E. coli*. The conserved region of *E. coli* SecEp is limited to the third putative membrane-spanning segment and the region immediately preceding it, exactly the domain that is essential for its function in protein translocation¹³. The putative SecEp homologues of other bacteria do not contain the preceding, non-essential sequences.

We have determined whether the mammalian Sec61- γ can functionally replace the yeast protein SSS1p *in vivo*. A diploid, heterozygous null mutant of *S. cerevisiae* was constructed in which one copy of *SSS1* was deleted (Δ sssl/SSS1). A multicopy plasmid was then introduced which carries the gene for the mammalian Sec61- γ under the control of the GAL promoter which can be induced by galactose and repressed by glucose. Tetrad analysis of this strain on glucose medium produced only two viable spores, confirming that SSS1 is an essential gene¹². Haploid cells carrying the deletion (Δ sssl) but containing the plasmid were viable on galactose (Fig. 3*a*, left) but not on glucose (Fig. 3*a*, right), indicating that lack of SSS1p can be compensated for by expression of Sec61- γ . The generation time on

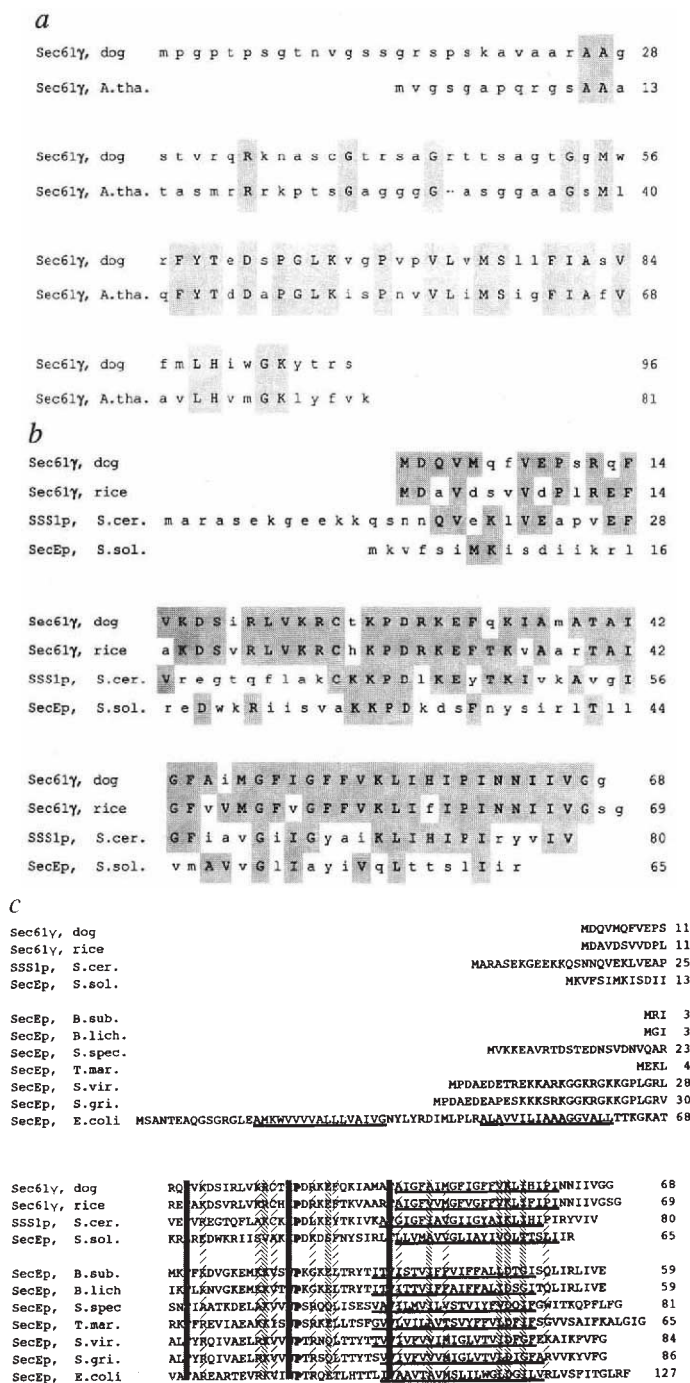


FIG. 2 Homologues of Sec61- β and Sec61- γ detected in the database. **a**, Comparison of the sequence of canine Sec61- β with that of its homologue in the plant *A. thaliana*. Identical residues are indicated in grey. **b**, Comparison of the sequence of canine Sec61- γ with that of its eukaryotic and archaeobacterial homologues. **c**, Multiple alignment of the eukaryotic/archaeobacterial Sec61- γ sequences with the eubacterial SecEp sequences. Positions of conservation are indicated by a hatched background, with the dark shading corresponding to those at which the fewest amino-acid exchanges occur. The conserved proline is indicated in bold.

METHODS. The search for homologous sequences was made using TBLASTN²¹ and TFASTA²². The following entries in GenBank were found: Sec61- β , A. tha., ATTS1834 (*A. thaliana*) (the sequence of the clone in the database was redetermined and corrected); Sec61- γ , rice, RIC1124A; SSS1p, S. cer., YSCAFR (*S. cerevisiae*); SecEp, S. sol., SSDOCK (*S. solfataricus*); SecEp, B. sub., BACSHRBPNS (*Bacillus subtilis*); SecEp, B. lich., BACSIGH (*Bacillus licheniformis*); SecEp, S. spec., SPMULTG (*Synechocystis spec.*); SecEp, T. mar., TMNUSG (*Thermogata maritima*); SecEp, S. vir., STMVBRA (*Staphylococcus viridiae*); SecEp,

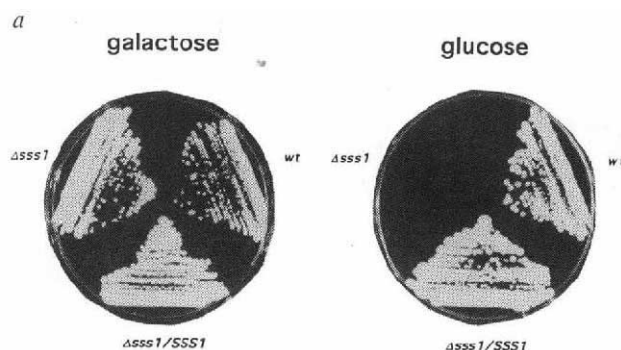
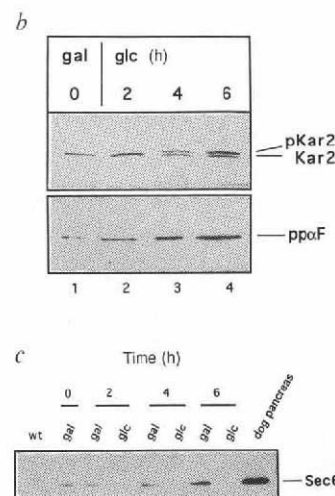


FIG. 3 Sec61- γ can functionally replace SSS1p in *S. cerevisiae*. **a**, A diploid, heterozygous deletion mutant (Δ SSS1/SSS1), a haploid wild type (wt) and a haploid deletion mutant (Δ SSS1), all carrying a multicopy plasmid with the mammalian SEC61- γ -gene under control of the GAL10 promoter, were tested for growth at 30 °C for 3 days on either galactose or glucose. **b**, A culture of Δ SSS1 cells was grown at 30 °C on galactose (gal) minimal medium to express Sec61- γ from a plasmid, and then shifted to glucose (glc). At the times indicated, the cells were labelled with ³⁵S-methionine for 10 min, proteins were extracted and immunoprecipitated with antibodies against KAR2 or α -factor.



antibodies against KAR2 or α -factor. The labelled proteins were separated by SDS-PAGE (12 and 18% acrylamide, respectively) and visualized by fluorography. pKAR2, ppaF, precursors to KAR2 and pro- α -factor, respectively. **c**, To follow the level of Sec61- γ , an experiment parallel to that in **b** was carried out, except that one portion of the cells continued growth on galactose. At different time points, cells were broken and crude membrane fractions were prepared, analysed by SDS-PAGE (7.5–17% acrylamide) and immunoblotted with antibodies specific for Sec61- γ . Positive and negative controls were carried out with membrane preparations from canine pancreas and wild-type yeast cells.

METHODS. The SSS1 gene was isolated by searching for multicopy suppressors of the sec61-2 ts-mutation (T.S. & S.J. unpublished data). The gene was disrupted by deleting the coding region between nucleotides –20–109 and replacing it by a 2.4-kb fragment carrying the ADE2 marker gene. A linearized plasmid containing Δ SSS1::ADE2 was transformed into a diploid wild-type strain (YTX69 mat a/a, homozygous his3-11, –15, leu2-3, –112, trp1-1, ura3-1, ade2-1, can1-100), resulting in a heterozygous mutant strain (YTX77, Δ SSS1::ADE2/SSS1). The complete coding region of Sec61- γ was amplified by the polymerase chain reaction at nucleotides 1–354 using specific oligonucleotide primers. The fragment was cloned behind the GAL10 promoter and the entire expression cassette was moved into the plasmid pRS424 (TRP1, 2 μ m)²⁵. Tetrad analysis, immunoprecipitation and immunoblot analysis were done^{5,26}. Antibodies against Sec61- γ were raised⁵ against a synthetic peptide corresponding to the first ten amino acids.

S. gri., SGNUSG (*Staphylococcus griseus*); SecEp, E. coli, ECOSECE (*E. coli*). A putative homologue of Sec61- β was also found in *S. solfataricus* (GenBank entry: SSORNPB). Partial sequences homologous to Sec61- γ found in the database: Sec61- γ mouse, MUSTUMSEQD; Sec61- γ , *C. elegans*, T01535; SecEp, *Thermus thermophilus*, S49804S1. Multiple alignment of the Sec61- γ /SecEp sequences was carried out using CLUSTAL²³. The statistical significance of the homologies was analysed with MACAW²⁴ using only one representative of highly related sequences and choosing as search lengths those of the individual proteins. The probability that the homology occurs by chance was estimated to be 3.3×10^{-16} .

galactose of the complemented $\Delta sss1$ strain was approximately equal to that of the wild type.

We also tested whether the translocation defect, which occurs upon depletion of SSS1p from yeast cells and is indicated by the accumulation of precursors of exported proteins¹², can be reversed by expression of Sec61- γ . Little accumulation of precursors to α -factor or Kar2p (BiP) was observed if the expression of Sec61- γ was induced in the presence of galactose (Fig. 3b, lane 1), indicating that the export of these proteins from the cytoplasm was close to normal. After shift to glucose, a severe defect of translocation developed (Fig. 3b, lanes 2–4), concomitant with the depletion of Sec61- γ from the cells (Fig. 3c, lanes 4, 6, 8). After 6 h, the cells ceased to grow (data not shown).

Signal sequences are similarly structured and exchangeable¹⁴ and one mechanism by which they are recognized—that involving the signal recognition particle—exists in all organisms^{15–19}. It is now clear that the component mediating the actual membrane passage of a polypeptide, the Sec61/SecYp complex, is also ubiquitous: two of its subunits, Sec61- α /SecYp and Sec61- γ /SSS1/SecEp, have been found in mammals, yeast and bacteria and, where tested, they are associated with each other. It remains to be seen whether Sec61- β , which so far has been found only in plants, also exists in yeast and whether it is related to the band-1 protein of the *E. coli* SecYp complex.

It is likely that Sec61- α /SecYp forms a channel that guides polypeptides across the membrane^{5,20}. Such a channel would be gated in two dimensions: perpendicular to the plane of the membrane to let polypeptides across, and horizontally within the membrane to release membrane-spanning regions of membrane proteins into the phospholipid bilayer and perhaps to let signal sequences in. Although the function of Sec61- γ /SSS1/SecEp is unknown, it may be involved in the second gating mechanism. Such a function would be consistent with the fact that this essential polypeptide, containing little more than a membrane-spanning region and a few adjacent residues, has remained separate from its multi-spanning partner during evolution. $\square$

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Interaction of *E. coli* Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor

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THE mechanism of protein translocation across the endoplasmic reticulum membrane of eukaryotic cells and the plasma membrane of prokaryotic cells are thought to be evolutionarily related^{1–7}. Protein targeting to the eukaryotic translocation apparatus is mediated by the signal recognition particle (SRP), a cytosolic ribonucleoprotein, and the SRP receptor, an endoplasmic reticulum membrane protein^{8,9}. During targeting, the 54K SRP subunit (*M*, 54,000; SRP54), a GTP-binding protein^{10–12}, binds to signal sequences^{13,14} and then interacts with the α -subunit of the SRP receptor (SR α), another GTP-binding protein^{12,15}. Two proteins from *Escherichia coli*, Ffh and FtsY, structurally resemble SRP54 and SR α ^{10,11,16}. Like SRP54, Ffh is a subunit of a cytosolic ribonucleoprotein that also contains the *E. coli* 4.5S RNA^{17,18}. Although there is genetic and biochemical evidence that the *E. coli* Ffh/4.5S ribonucleoprotein has an SRP-like function^{19–21}, there is no evidence for an SR α -like role for FtsY. Here we show that the Ffh/4.5S ribonucleoprotein binds tightly to FtsY in a GTP-dependent manner. This interaction results in the stimulation of GTP hydrolysis which can be inhibited by synthetic signal peptides. These properties mimic those of mammalian SRP and its receptor, suggesting that the *E. coli* Ffh/4.5S ribonucleoprotein and FtsY have functions in protein targeting that are similar to those of their mammalian counterparts.

To test for an interaction between the Ffh/4.5S ribonucleoprotein (RNP) and FtsY, Ffh and 4.5S RNA were purified from overproducing strains and reconstituted into an RNP. FtsY was purified as a fusion protein with glutathione *S*-transferase (FtsY-GST) and immobilized on a glutathione-affinity resin, which was then incubated with Ffh/4.5S RNP in the presence of either GDP or the non-hydrolysable GTP analogue GMP-PNP. Nucleotide was included in these reactions because both Ffh and FtsY contain GTP-binding domains which might regulate their interaction. More than 90% of the Ffh co-fractionated with the FtsY-GST resin in the presence of GMP-PNP; most of the Ffh was recovered in the supernatant fraction in the presence of GDP (Fig. 1). Similarly, most of the Ffh was recovered in the supernatant fraction in the presence of the non-hydrolysable ATP analogue AMP-PNP (not shown), indicating that the interaction requires guanosine triphosphate. Free Ffh protein failed to bind to FtsY-GST resin even in the presence of GMP-PNP (Fig. 1), indicating that FtsY binding requires both Ffh and 4.5S RNA. Neither Ffh/4.5S RNP nor free Ffh bound to a control resin containing immobilized GST, indicating that binding is specific for FtsY (Fig. 1).

Interestingly, no binding was observed when the reaction shown in Fig. 1 was performed with GTP instead of GMP-PNP (not shown), indicating that GTP might be hydrolysed during the reaction. We therefore analysed the ability of these components to hydrolyse GTP. As shown in Fig. 2, purified Ffh hydro-

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FIG. 1 Ffh/4.5S binds to immobilized FtsY-GST in the presence of GMP-PNP. Purified Ffh protein and Ffh/4.5S RNP were assayed for their ability to bind to FtsY-GST or GST immobilized on glutathione-agarose beads in the presence of either GDP or GMP-PNP. After binding, the beads were collected by centrifugation, and the proteins recovered in the pellet (p) and supernatant (s) fractions were analysed on SDS-polyacrylamide gels and visualized by staining with Coomassie blue. The amount of Ffh protein in each lane is expressed as a percentage of the total Ffh protein recovered in pellet and supernatant fractions. **METHODS.** Ffh protein and 4.5S RNA were prepared as described^{17,21}. The FtsY-GST fusion protein was produced by fusing amino acids 48–494 of FtsY in-frame to the C terminus of GST in the pGEX1 vector²⁷. The region of strong homology between FtsY and SR α encompasses amino acids 197–494 and includes the GTP-binding domain. Purified FtsY-GST fusion protein (10 pmol) or GST protein was incubated under constant mixing with 50 μ l of a 50% suspension of glutathione-agarose beads (Sigma) in phosphate-buffered saline for 1 h at room temperature. The resin was washed with buffer A containing 50 mM triethanolamine/acetate (TEA), pH 7.5, 25 mM potassium acetate, 2.5 mM Mg(OOC·CH₃)₂, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nikkol detergent (Nikko Chemicals, Tokyo) and then added to 100 μ l of the same buffer containing 5 pmol of Ffh or Ffh/4.5S RNP. GDP or GMP-PNP was added to a final concentration of 1 mM where indicated. Reactions were incubated with constant mixing at room temperature for 30 min. The resin was pelleted by centrifugation in a microfuge for 30 s and the supernatant removed. Proteins in the supernatant were precipitated with an equal volume of 30% trichloroacetic acid and solubilized in SDS sample buffer. The resin was washed with buffer A containing either GDP or GMP-PNP at 1 mM, and bound proteins were then solubilized by boiling the beads in SDS sample buffer. Quantification was by laser scanning densitometry of the stained gel. The Ffh/4.5S RNP was prepared by incubating a solution containing 10 μ M Ffh and 20 μ M 4.5S RNA in buffer B containing 50 mM TEA, 500 mM potassium acetate, 5 mM Mg(OOC·CH₃)₂, 1 mM DTT, 0.01% Nikkol and 10% glycerol for 10 min on ice followed by 10 min at 37 °C.

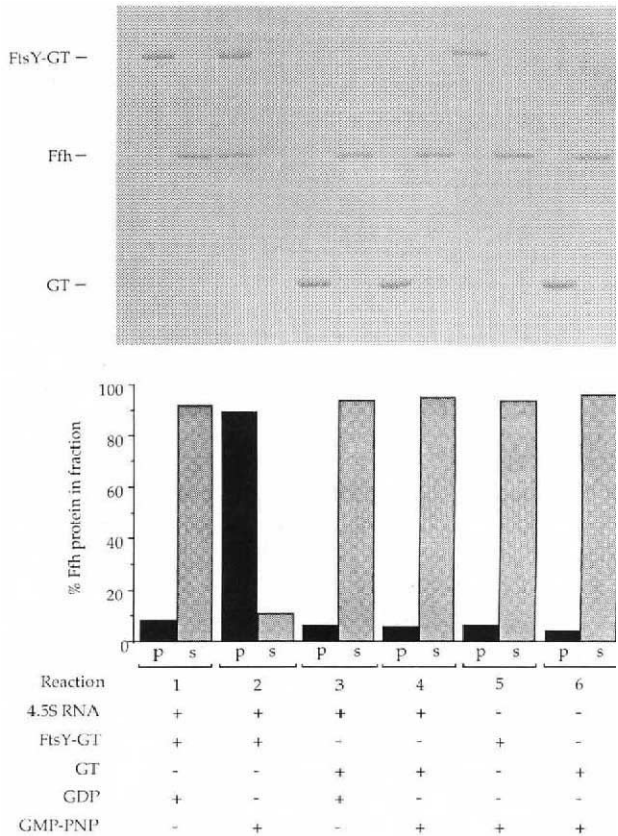


FIG. 2 Enhanced GTP hydrolysis results from interaction of Ffh/4.5S RNP and FtsY. Purified Ffh/4.5S RNP and purified Ffh protein were assayed for the ability to hydrolyse GTP in the presence or absence of FtsY-GST or GST. **METHODS.** Reactions (100 μ l) were incubated in buffer A containing 10% glycerol. Ffh/4.5S RNP complexes were formed as described in Fig. 1 legend. Ffh was present at 5 nM, 4.5S RNA at 10 nM, FtsY-GST and GST at 150 nM. [γ -³²P]GTP (0.5 μ Ci; ICN) was added at 0.5 μ M. After a 20-min incubation at 25 °C an 8- μ l aliquot of the reaction was mixed with 200 μ l of a 5% suspension of activated charcoal (Sigma) in 20 mM phosphoric acid, incubated on ice for 10 min and then centrifuged for 10 min at maximum speed in a microfuge to pellet the charcoal and bound nucleotide. A 100- μ l aliquot of the supernatant fraction containing the phosphate liberated in the reaction was analysed by Cerenkov counting in a liquid scintillation counter and corrected for background counts from a reaction performed with buffer only. The Michaelis constant of the Ffh/4.5S RNP for GTP hydrolysis was 0.4 μ M; the rate of hydrolysis was linear during the 20-min incubation.

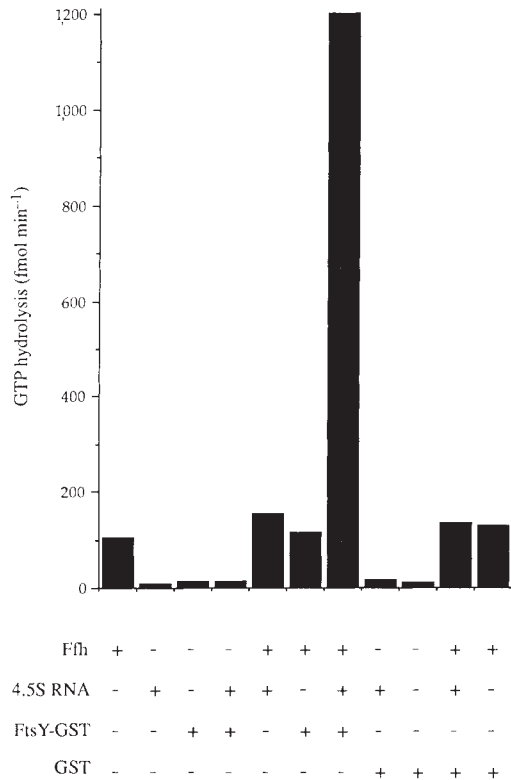
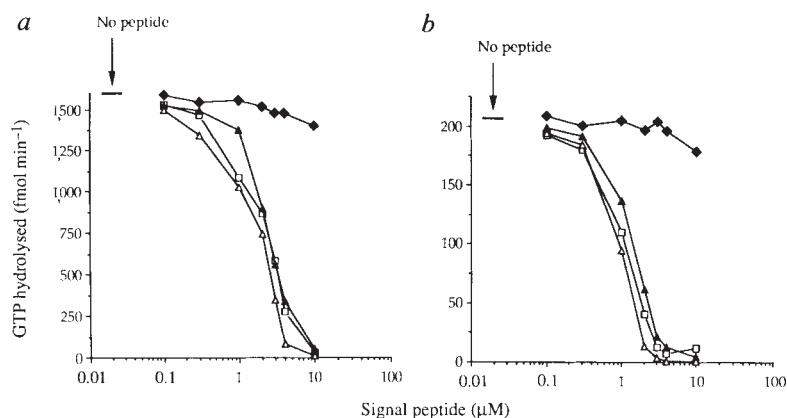


FIG. 3 Synthetic signal peptides inhibit GTP hydrolysis. **a**, Effect of titrating synthetic signal peptides into GTP hydrolysis reactions containing Ffh/4.5S RNP and FtsY. **b**, Effect of titrating signal peptide into GTP hydrolysis reactions containing Ffh/4.5S RNP in the absence of FtsY. Reactions are described in Fig. 2 legend, except that reaction mixtures were preincubated for 30 min at 25 °C with the appropriate synthetic signal peptide before addition of GTP. Four different peptides were used: wild-type peptide ($\square$); sequence MMITLRKLPLA-VAVAAGVMSAQAMA, corresponding to the signal sequence of LamB, a bacterial outer-membrane protein; deletion mutant peptide ($\blacklozenge$), corresponding to the Lam B signal sequence with residues L10-A13 deleted; r1 ($\blacktriangle$) (corresponding to the deletion mutant peptide with a G17C change) and r2 ($\triangle$) (corresponding to the deletion mutant peptide with a P9L change) peptides, which correspond to second-site, single-amino-acid revertants of the deletion mutant. The synthetic wild-type peptide can readily adopt an α -helical conformation as analysed by circular dichroism spectroscopy²⁴. The missing four amino acids in the deletion mutant peptide cause a proline and a glycine residue to be brought closer so



that these two residues function as helix breakers. The synthetic deletion mutant peptide does not form an α -helix²⁴, and does not function as a signal peptide *in vivo*²³. Both r1 and r2 peptides regain the ability to form an α -helix and function as signal sequences *in vivo*^{23,24}.

lysed GTP, and the rate of hydrolysis was independent of the presence of 4.5S RNA. The enzymatic properties of this reaction closely resemble those previously described for an Ffh homologue from *Mycoplasma mycoides*²². In contrast, FtsY-GST caused no significant GTP hydrolysis (Fig. 2). When the Ffh/4.5S RNP and FtsY-GST were combined, however, GTP hydrolysis was significantly stimulated. The stimulation requires that Ffh be complexed to 4.5S RNA; in the absence of the RNA only the basal, Ffh-catalysed hydrolysis rate was observed. No stimulation of GTP hydrolysis was observed when GST was incubated with Ffh/4.5S RNP, indicating that the activity is specific for FtsY (Fig. 2). Together with the binding data shown in Fig. 1, these results provide strong evidence for a functional interaction between Ffh/4.5S RNP and FtsY.

As signal sequences are possible physiological ligands of the Ffh/4.5S RNP^{20,21}, we tested whether the binding of synthetic signal peptides could modulate GTP hydrolysis. We assayed the effects of peptides derived from the signal sequence of LamB, a bacterial outer-membrane protein that exhibits a translocation defect in Ffh-depleted cells¹⁹. Four different peptides were used: a peptide corresponding to the wild-type signal sequence, a deletion mutant that is inactive *in vivo*, and two different single-amino-acid, second-site revertants of the LamB deletion mutant (r1 and r2), both of which restore signal-sequence function^{23,24}. The wild-type, r1 and r2 synthetic peptides, but not the deletion mutant peptide, inhibit *in vitro* protein translocation into *E. coli* inverted plasma membrane vesicles²⁵.

The data shown in Fig. 3a indicate that the wild-type signal peptide potentially inhibits the FtsY-GST-stimulated GTP hydrolysis reaction with a half-maximal inhibitory concentration (IC_{50}) of $\sim 2 \mu M$. In contrast, the deletion mutant peptide had little effect on GTP hydrolysis even at concentrations at which the wild-type peptide inhibited completely. Both reversion mutations, r1 and r2, restored the inhibitory activity to the deletion mutant peptide to about the same level as that exerted by the wild-type peptide. Therefore only peptides corresponding to functional signal sequences (namely wild-type, r1 and r2) inhibit GTP hydrolysis.

Examination of the inhibition curves reveals that the functional signal peptides block GTP hydrolysis completely, so FtsY-stimulated hydrolysis and basal hydrolysis catalysed by Ffh alone were both blocked. This was confirmed in experiments shown in Fig. 3b, in which the same set of peptides were titrated into a reaction containing only Ffh. The functional peptides inhibited the GTPase activity of Ffh with a concentration dependence similar to that of the FtsY-stimulated reaction (Fig.

3b). Whether Ffh, FtsY or both hydrolyse GTP in the FtsY-stimulated reaction remains to be determined.

These results provide evidence that the SRP receptor-like FtsY protein interacts directly with the SRP-like Ffh/4.5S RNP. The interaction results in enhanced GTP hydrolysis that can be modulated by signal sequences. The biochemistry of the interaction of the Ffh/4.5S RNP with FtsY and with two other ligands, signal sequences and GTP, is remarkably similar to that found for the corresponding mammalian components. In particular, SRP and its receptor form a stable complex in the presence of GMP-PNP²⁶. Furthermore, SRP receptor and SRP54 interact to hydrolyse GTP in an SRP RNA-dependent manner, and functional signal sequences inhibit this reaction with comparable IC_{50} s (ref. 12). This striking similarity supports the hypothesis that bacteria have a signal-recognition and protein-targeting apparatus composed of the Ffh/4.5S RNP and FtsY which resembles the SRP and SRP receptor system in eukaryotic cells. $\square$

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Measurement of the β -sheet-forming propensities of amino acids

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SEVERAL model systems have been used to evaluate the α -helical propensities of different amino acids^{1–7}. In contrast, experimental quantitation of β -sheet preferences has been addressed in only one model system, a zinc-finger peptide⁸. Here we measure the relative propensity for β -sheet formation of the twenty naturally occurring amino acids in a variant of the small, monomeric, β -sheet-rich, IgG-binding domain from protein G. Amino-acid substitutions were made at a guest site on the solvent-exposed surface of the β -sheet. Several criteria were used to establish that the mutations did not cause significant structural changes: binding to the Fc domain of IgG, calorimetric unfolding and NMR spectroscopy. Characterization of the thermal stabilities of these proteins leads to a thermodynamic scale for β -sheet propensities that spans a range of ~ 2 kcal mol^{–1} for the naturally occurring amino acids, excluding proline. The magnitude of the differences suggests that β -sheet preferences can be important determinants of protein stability.

The immunoglobulin-binding domain B1 from protein G (denoted GB1) is a small, soluble, monomeric, highly stable protein comprising a four-stranded β -sheet and a single α -helix (Fig. 1a). GB1 has been shown to undergo a two-state, reversible, thermal unfolding transition^{9,10}. These properties make GB1 an attractive model system for studying β -sheet propensities. A 'guest' site, into which all twenty natural amino acids could be substituted, was chosen at a solvent-exposed position (residue 53) in a central β -strand of the protein. This position was chosen to avoid potential effects from the edges or ends of the sheet (Fig. 1a).

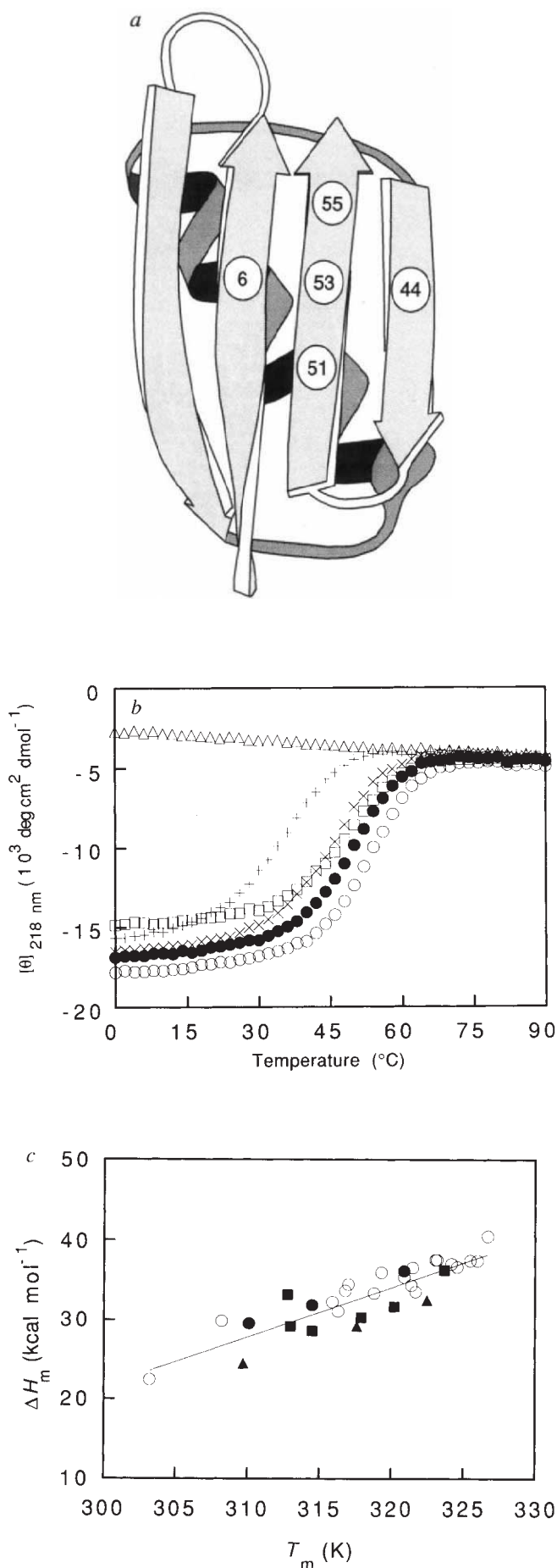
As we wanted to measure the intrinsic propensities for β -sheet formation, we constructed potential host molecules that minimized local interactions to the guest site. Three potential host systems were constructed in which the guest position was surrounded by small neutral amino acids. The majority of inter-residue contacts to the guest site arise from the two residues on each of the immediately adjacent β -strands (Fig. 1a). These residues, Ile 6 and Thr 44, were replaced by alanine. The residues $i-2$ and $i+2$ from the guest site (residues 51 and 55) make fewer, more distant contacts and were replaced simultaneously with alanine, serine or threonine.

Glycine, an amino acid expected to have a low β -sheet propensity^{8,11}, was substituted at the guest site in order to evaluate the potential host systems. In the system containing alanine substitutions at the $i-2$ and $i+2$ positions, with glycine at the guest position, the circular dichroism (CD) thermal unfolding curve lacks a clearly defined folded baseline (unpublished results). A well-defined folded baseline, however, is obtained with the glycine mutant in the systems containing serine or threonine substitutions at the $i-2$ and $i+2$ positions. The host containing serine, the smaller side-chain substitution, at the $i-2$ and $i+2$ positions (I6A/T44A/T51S/T55S; denoted AASS) was chosen for further study.

The twenty naturally occurring amino acids were substituted for residue 53 in the AASS background by site-directed mutagenesis. The stability of each protein (designated AASS-53Xaa) was measured by thermal unfolding as monitored by CD (Fig. 1b). $\Delta\Delta G$ values for β -sheet formation were obtained by assuming that changes in global stability result entirely from changes

FIG. 1 a, Ribbon drawing of GB1 (based on a diagram produced with the program RIBBON¹⁷ using the coordinate set 2GB1¹⁰). The positions of the guest site and the surrounding residues are indicated. The arrows represent the inter-residue side-chain–side-chain contacts to the guest site made by the surrounding residues. In the native structure of GB1, the number of contacts to residue 53 from residues 6, 44, 51 and 55 is twelve, seven, three and two respectively. In the AASS background the number would be reduced to three, two, one and two respectively, assuming that the backbone structure is unperturbed from that of GB1. b, Temperature dependence of the CD signal from representative GB1 mutants. AASS-53Thr (○), AASS-53Ser (●), AASS-53Gln (×), AASS-53Asp (+), AASS-53Trp (□), AASS-53Pro (△). The folded baselines of all molecules fall within the bounds of the range shown here, with the exception of AASS-53Gly, which has a $[\theta]_{218\text{nm}}$ value of $-13,100$ deg cm² dmol^{–1} at 0 °C. The differences in folded baseline $[\theta]_{218\text{nm}}$ values probably result largely from different aromatic contributions to the CD signal. Residue 53 is situated above W43, which is in the hydrophobic core of the protein. Molecules containing the mutation W43F show changes in both the folded and unfolded $[\theta]_{218\text{nm}}$ baseline values of 45 and 35% respectively (data not shown), indicating that W43 is making a substantial contribution to the CD signal at 218 nm (see also text and refs 13, 14). c, Plot of ΔH_m versus temperature for the AASS mutants. Open circles indicate T_m and ΔH_m values from the AASS-53Xaa mutants. Filled symbols indicate T_m and ΔH_m for AASS-53Thr (●), AASS-53Phe (▲) and AASS-53Val (■), at different pH values. The slope of the line is ΔC_p (624 cal mol^{–1} deg^{–1}).

METHODS. Calculations of nearest-neighbour side-chain contacts were made using a program written by T. G. Oas¹⁸. Significant contacts were identified from the coordinate set 2GB1 and were defined as any atom pair having a centre-to-centre distance less than or equal to 150% of the sum of the van der Waals radii. A synthetic gene for the GB1 sequence¹⁹ was constructed and cloned into a T7 expression plasmid²⁰, pAED4 (ref. 21), using standard cloning procedures²². All proteins were purified by affinity purification⁹ on IgG 6 Fast-Flow Sepharose (Pharmacia, 17-0969-01) followed by reverse-phase high-pressure liquid chromatography (HPLC) purification on Vydac preparative C18 column using a linear H₂O-acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. This purification scheme yielded two species of GB1, a 56-residue protein containing an N-terminal methionine and a 55-residue protein in which the N-terminal methionine had been removed. The 55-residue species was substantially less stable (by ~ 1.7 kcal mol^{–1}) than the 56-residue species. The mutation Met1 $\rightarrow$ Thr was made to produce a 56-residue, N-terminally processed protein that began with threonine at position 1. This protein had the same stability as the 56-residue GB1. All mutant genes were made in the GB1 background containing threonine at position 1 by site-directed mutagenesis²³. Proteins were purified by affinity and HPLC chromatography as described, except for AASS-53Pro, for which the IgG affinity-column step was replaced by G75 chromatography in a buffer of 10 mM phosphate, 150 mM NaCl, pH 7.3. Mutant genes were sequenced completely (Sequenase, USB) and the identity of each HPLC-purified protein was confirmed by laser desorption mass spectrometry (Finnegan Mat Lasermat). All relative molecular masses were within 3 units of the expected M_r . CD measurements were made at a concentration of 10 μ M using a 1-cm pathlength cell with an Aviv model 62DS circular dichroism spectrometer equipped with a thermoelectric temperature controller. Sample buffer contained 50 mM CH₃COO Na, 150 mM NaCl, pH 5.4. The melting curves were fitted (Kaleidagraph; Abelbeck Software) to the equation $\theta = \theta_u + (\theta_f - \theta_u) / (1 + e^{((-\Delta H_m/R)(1/T - 1/T_m) + (\Delta C_p/R)[((T_m/T) - 1) + \ln(T/T_m))])}$, using a fixed ΔC_p of 624 cal mol^{–1} deg^{–1}, where θ is the measured CD signal, θ_f and θ_u are linear functions representing the folded and unfolded baselines respectively, T_m is the melting temperature, and ΔH_m is the enthalpy of unfolding at T_m (ref. 24). The two-state unfolding model was tested for AASS-53Thr and AASS-53Ala by measuring the enthalpy of unfolding by differential scanning calorimetry. The results are $\Delta H_{cal} = 39.9$ kcal mol^{–1} and 32.8 kcal mol^{–1}, $\Delta H_{cal}/\Delta H_{van't Hoff} = 0.99$ and 0.97 for AASS-53Thr and AASS-53Ala respectively. Differential scanning calorimetry experiments were performed using a Microcal MC-2 scanning calorimeter (Northampton, MA). Calorimetry samples contained ~ 3 mg ml^{–1} protein in 50 mM sodium acetate, 150 mM NaCl, pH 5.4, and were dialysed extensively against this buffer before the experiment. Samples were degassed under vacuum before loading into the sample chamber and were heated from 4 to 95 °C using a scanning rate of one deg per min. The pH dependence of T_m was measured in a buffer of 1 mM each of phosphate, acetate, citrate and borate in 196 mM NaCl. Protein concentration was determined for all samples from the absorbance of the unfolded protein²⁵.



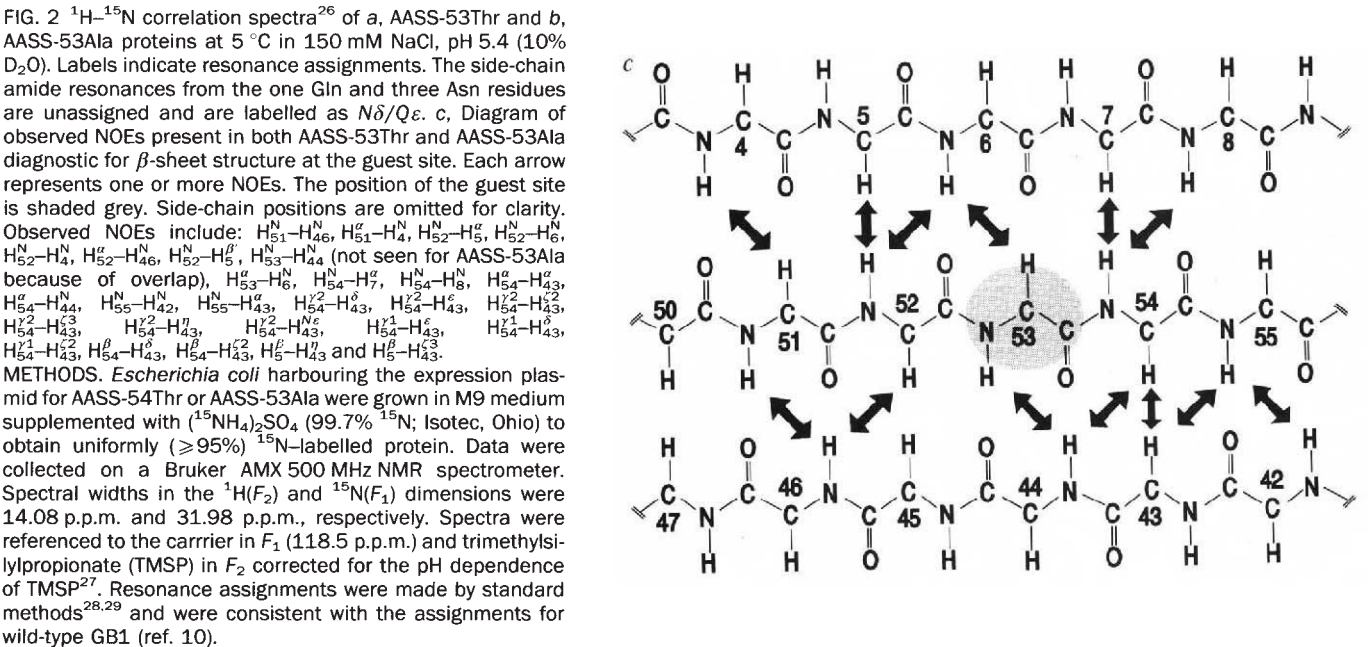
in the ability of the residue at the guest site to adopt a β -sheet conformation. ΔC_p for unfolding, a necessary parameter for calculating the temperature dependence of the free energy of unfolding, was determined by combining data from the pH dependence of stability for AASS-53Thr, AASS-53Phe and AASS-53Val with the stability data obtained at pH 5.4 for each of the guest site mutants (Fig. 1c). The resultant value ($\Delta C_p = 624 \pm 62 \text{ cal mol}^{-1} \text{ deg}^{-1}$) is in excellent agreement with the previously reported value for wild-type GB1 ($621 \pm 71 \text{ cal mol}^{-1} \text{ deg}^{-1}$)⁹ and these data illustrate that these mutations have little effect on ΔC_p , as might be expected for mutations at solvent-exposed positions¹². The relative free energy differences for β -sheet formation are listed in Table 1.

There are differences among the mutant proteins in the magnitude of the CD signal at 218 nm (Fig. 1b). However, all the

TABLE 1 $\Delta\Delta G$ values for β -sheet formation relative to alanine, thermal melting temperatures (T_m), and relative binding constants to human Fc for the AASS-53Xaa proteins normalized to the binding of AASS-53Thr

Amino acid	$\Delta\Delta G$ (kcal mol ⁻¹)	T_m (°C)	$K_a/K_a^{\text{AASS-53Thr}}$
Thr	1.1	53.7	1.0
Ile	1.0	53.0	1.0
Tyr	0.96	52.5	1.0
Phe	0.86	51.6	1.0
Val	0.82	51.2	1.1
Met	0.72	50.2	1.0
Ser	0.70	50.1	1.1
Trp	0.54	48.7	1.1
Cys	0.52	48.5	1.1
Leu	0.51	48.4	1.0
Arg	0.45	47.9	1.0
Lys	0.27	46.3	1.1
Gln	0.23	45.8	1.2
Glu	0.01	44.0	1.1
Ala	0.00	43.8	1.1
His	-0.02	43.3	1.0
Asn	-0.08	42.9	1.0
Asp	-0.94	35.2	1.1
Gly	-1.2	30.2	1.0
Pro	< -3	< 0	0

The K_a for wild-type GB1 is $1.4 \times 10^8 \text{ M}^{-1}$ (ref. 15). The $K_a/K_a^{\text{AASS-53Thr}}$ value for wild-type GB1 is 1.1. ΔG values for unfolding of the AASS-Xaa proteins were calculated using the Gibbs-Helmholtz equation $\Delta G(T) = \Delta H_m (1 - T/T_m) - \Delta C_p [T_m - T + T \ln(T/T_m)]$, assuming $\Delta C_p = 624 \text{ cal mol}^{-1} \text{ deg}^{-1}$ for all molecules (Fig. 1c). $\Delta\Delta G$ values were calculated at 321 K, the mean of the T_m s excluding the glycine mutant, to minimize extrapolations in the calculation of $\Delta G(T)$. A positive value of $\Delta\Delta G$ indicates an increase in stability. The estimated errors in determination of T_m and ΔG are $\pm 0.5^\circ \text{C}$ and $\pm 0.06 \text{ kcal mol}^{-1}$ respectively. Fc was made by proteolytic digestion of human IgG (Sigma I-4506) with papain and was purified from other digestion products by affinity (protein G)¹⁶ and gel-filtration (G75) chromatography in a buffer of 10 mM phosphate, 150 mM NaCl, pH 7.3, followed by FPLC purification using a linear gradient of NaCl (0.05–1 M) in a buffer of 50 mM sodium acetate, pH 4.75, on Mono-S resin (Pharmacia). The purified material was $>98\%$ pure as judged by SDS polyacrylamide gel electrophoresis. The binding assay was carried out using standard methods¹⁶ on 96-well microtitre-plates (Costar), coated for 2 h with a $100 \mu\text{g ml}^{-1}$ solution of Fc and then blocked for 2 h with 3% bovine serum albumin (BSA). The assay was carried out at 5°C using a fixed quantity of protein G-alkaline phosphatase ($100 \mu\text{g ml}^{-1}$) (Pierce 31399X) and a variable amount of competitor protein (0.6–3.5 mM) in the presence of 1.5% BSA in a buffer of 50 mM sodium acetate, 150 mM NaCl, pH 5.4. Samples were allowed to equilibrate for 39–48 h. Control experiments indicated that equilibrium was reached after $\sim 25 \text{ h}$. Wells were developed with a fresh solution of 1 mg ml^{-1} *p*-nitrophenol phosphate (Pierce 34045X) and the absorbance at 410 nm was read using a microplate reader (Dynatech). The ratio of affinity constants of the competitor molecule with protein G was determined using the equation $(Y \times Y)/(Y - 1) = -(K_a^{\text{protein G}}/K_a^{\text{competitor}})[\text{competitor}]$, where Y is the fraction of protein G-alkaline phosphatase bound. The ratio of affinity constants for the mutants was normalized to AASS-53Thr, which was run as an internal standard on each plate.



Two molecules representing each end of the stability scale, AASS-53Thr and AASS-53Ala, were chosen for further charac-

AASS-53Thr and AASS-53Ala were also investigated by NMR. The ^1H - ^{15}N correlation spectra of these two molecules are very similar except for resonances from residues in the immediate vicinity of the guest site (Fig. 2*a, b*). The NMR spectra of both proteins also contain cross-strand nuclear Overhauser effect (NOE) patterns which are present in wild-type GbI and are

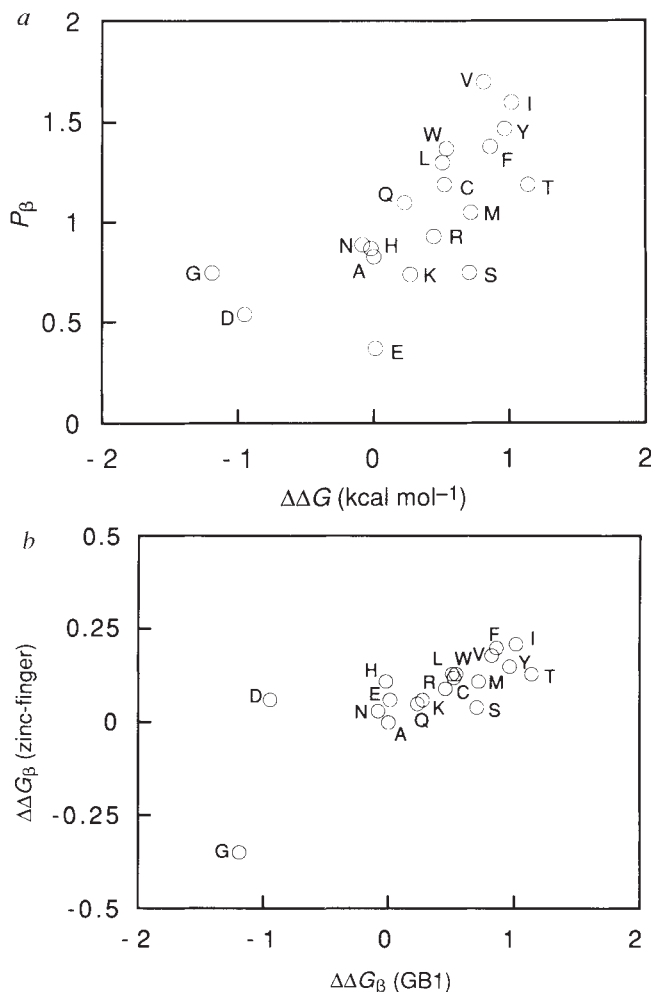


FIG. 3 *a*, Correlation of the $\Delta\Delta G$ values for β -sheet formation measured in the AASS system (Table 1) with the β -sheet-forming propensities (P_β) of Chou and Fasman¹¹. *b*, Correlation of the $\Delta\Delta G$ values for β -sheet formation obtained here and in the zinc-finger system⁸. Note the difference in scale between the two systems. The value for proline is omitted.

indicative of β -sheet structure at the guest site (Fig. 2c). In particular, these data suggest that any structural changes between the mutants are small and do not involve significant disruption of the backbone β -sheet structure, such as fraying of residues 53–56.

Our results indicate that there are significant differences in β -sheet-forming propensities among the naturally occurring amino acids. The propensity scale suggests that β -branching of the side chain favours β -sheet formation, and the rank order shows a modest correlation with β -sheet preferences measured by statistical methods¹¹ and with the rank order measured in the zinc-finger system⁸ (Fig. 3). It is striking, however, that the range of $\Delta\Delta G$ values for the β -sheet formation obtained here is an order of magnitude larger than the values obtained with the zinc-finger host (2.05 kcal mol⁻¹ versus 0.21 kcal mol⁻¹ full scale, excluding proline and glycine).

Finally, the magnitude of β -sheet preferences obtained here is comparable to those measured for α -helices^{1,7}, suggesting that β -sheet propensities can be as important as α -helix propensities in determining protein stability. The thermodynamic preferences for β -sheet formation reported here should be useful for deciphering the rules involved in protein stability, protein folding and protein design.

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Separation protocols

This week's line-up includes a blood cell agglutination reagent, a kit for isolating immunoglobulin from egg yolk, a capillary electrophoresis system and a gas chromatography system that provides qualitative structural information.

RED-OUT, a new **blood cell agglutination reagent** from Robbins Scientific, removes human red cells from centrifugally separated lymphocytes (*Reader Service No. 100*). The specificity of the reagent is based on a murine monoclonal antibody that binds to a universally present antigen on erythrocyte membranes. Red-Out can be used for the removal of red cells, including immature nucleated erythrocytes, that can interfere in a number of applications (HLA tissue typing tests, and microscopic and fluorescence-activated cell sorting). Two sizes are available providing sufficient reagent for 250 or 1,000 tests.

A recombinant form of peptide-*N*-glycosidase F, an **enzyme isolated from the bacterium *Flavobacterium meningosepticum***, can now be obtained from Oxford GlycoSystems (*Reader Service No. 101*). Stated applications include the controlled recovery of de-*N*-glycosylated peptides and proteins, selective recovery of intact *N*-linked oligosaccharides from glycoproteins and use in methods to probe the biological significance and functional roles of *N*-glycosylation. The recombinant form of the enzyme, which is overexpressed in *Escherichia coli*, is said to offer several advantages over the native form, namely high batch-to-batch consistency and a lower price. It is purified to remove proteases and contaminating glycosidase activities. The enzyme preparation is then screened for contaminating activities against both synthetic chromogenic substrates and a range of naturally occurring oligosaccharides. It is supplied with a glycerol-free incubation buffer.

Middlesex Sciences introduces a new **method for obtaining pure immunoglobulin from egg yolk** (*Reader Service No. 102*). The company says that its new gamma Yolk antibody purification kit can be used to obtain 75–100 mg IgY per egg. The kit utilizes polymer reagents to form soluble complexes with contaminating proteins, including albumin and lipoproteins. With these molecules held in solution, target immunoglobulin is selectively precipitated out. The protocol is said to take 4 h, with only 30 min of hands-on time.

■ Electrophoresis

The P/ACE 5000 series **capillary electrophoresis systems** from Beckman Instruments feature a new diode array, which incorporates a high-resolution array and covers the range from 190 to 600 nm, and selectable ultraviolet absorbance detectors (*Reader Service No. 103*). Complementing



Beckman's P/ACE 5000 series features selectable UV absorbance detectors and a new diode array detector.

the new system is the latest revision of Beckman Gold software, which provides spectral scanning, derivative calculations, spectral subtraction and real-time peak purity monitoring. The Array View and QuickRes* (*Infometrics) software options generate three-dimensional plots, isoabsorbance maps and electropherograms at any wavelength, in addition to resolving co-eluting peaks. Options such as the laser-induced fluorescence detector and mass spectrometry adapter operate with the 5000 series with no modification.

The new NucleoPhor SB1.5 kb **sieving buffer kit** from Dionex combines capillary and buffer systems specifically designed to separate double-stranded nucleic acids from 30 to 1,500 base-pairs long, including PCR



Dionex's capillary electrophoresis method for DNA separations.

products and restriction fragments, while providing automated sample handling and online detection (*Reader Service No. 104*). The kit is optimized for use at ambient temperatures, buffers are supplied ready to use, sample pretreatment is not necessary and direct detection eliminates labelling agents. Each kit consists of a DNA standard, mobility markers and enough capillaries and buffer for more than 400 runs.

AT Biochem's new 64-page **catalogue and handbook** provides an extensive selection of products for electrophoresis (*Reader Service No. 105*). Featured new products include HydroLink gels, including Long-Ranger for DNA sequencing, MDE for the detection of mutations, PCR Purity Plus for PCR analysis and ProRanger for protein separations. Other highlights include DNA and protein molecular weight markers, oligonucleotides, gel analysis software, apparatus for electrophoresis, and a range of reagents and accessories. Protocols, notes on new applications, references and technical information are also included.

■ Stains and standards

Zoion Biotech's new product, QuickStain, is a **negative stain for protein** for use in polyacrylamide gel electrophoresis (PAGE and SDS PAGE) and for membrane-based staining procedures (*Reader Service No. 106*). Staining procedures can be completed in about 20 min and do not involve the use of fixatives or protein denaturing agents. Proteins are said to be recovered in a fully active form and are antigenically intact after staining. The stain can also be used with membrane-based protein sequencing applications.

The SeeBlue **pre-stained, wide-range protein standard** developed by Novex, which is available from R & D Systems, allows you to monitor continuously gel runs, providing a useful means of assessing the quality of western blot transfers (*Reader Service No. 107*). The standard contains nine proteins ranging from 4 to 250 kilodaltons for the calibration of all types of SDS gels (4–20 per cent); it can also be used for gradient gels. When electrophoresed, proteins are said to form sharp, uniformly stained blue bands. R & D Systems says the bands remain sharp and as intense, even after Coomassie staining. No prior mixing or diluting of the standard is necessary.

The new Kaleidoscope **prestained standards** from Bio-Rad are designed to provide researchers with a quick and easy way to assess the quality of an electrophoretic transfer and serve as a control in repetitive blotting experiments (*Reader Service No. 108*). Individual bands are identified by their different colours, making it possible to monitor the separation of proteins while the run is in progress — even after the dye front has run off the gel. The standards can also be used to locate a protein for excision from an unstained preparative gel. Kaleidoscope standards consist of seven coloured proteins with a molecular weight range of about 6,500–200,000

Three new products from Genomed focus on isolation, **purification and concentration of double-stranded nucleic acids** (*Reader Service No. 109*). Jetsorb can be used to extract 20 base pairs to 45 kilobase DNA fragments from all types of agarose gels, including Tris-Borate-EDTA gels. Jetpure, on the other hand, has been developed for the separation of double-stranded DNA from single-stranded oligonucleotides, polymerases and proteins. Completing the line-up is Jetnick, which can be used for the purification of DNA fragments after *in vitro* labelling reactions. All three are said to provide 85–95 per cent recovery for the extraction of 20 bp to 45 kb DNA fragments. Genomed is expected to release Jetprep, a mini-prep plasmid DNA isolation kit, in the near future.

By combining three Takara products, Pan Vera is able to offer a one-hour **protocol for subcloning** that consists of a six-step process (*Reader Service No. 110*). Step 1 uses the Suprec-01 cartridge, which is designed for the recovery of DNA from agarose gel slices. An excised band is placed in the cartridge and centrifuged for 5 min. The recovered DNA is then available for subsequent enzymatic reactions and cloning procedures. Step 2 involves placing the filtrate from the previous step in the Suprec-02 cartridge and centrifuging for a further 2 min. The purified and concentrated DNA is then retained on the filter membrane. Suprec-02 cartridges can also be used to purify DNA amplified using PCR, for buffer exchange, and to eliminate unused nucleotides after labelling reactions. The DNA is then suspended in Tris-EDTA buffer and combined with the appropriate vector for ligation. Solutions from the DNA ligation kit are added and ligation is allowed to proceed for

15 min. Step 3 uses the DNA ligation kit to perform ligations in 30 min. After the ligation is complete, an aliquot of the ligation mixture is combined with the appropriate competent cells and incubated on ice for 15 min at 37 °C (steps 4 and 5). Step 6 involves plating of the cells.

Millipore's new Ultrafree-MC DEAE **centrifugal filter device** is intended for small-volume, centrifugal anion-exchange applications (*Reader Service No. 111*). The filter



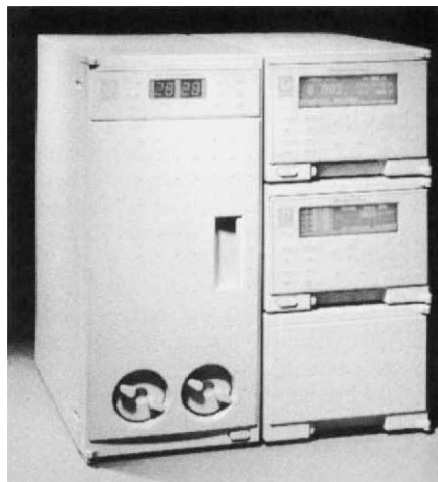
Small-volume filtration devices.

device, which contains a microporous regenerated cellulose membrane derivatized with diethylaminoethyl (DEAE) anion-exchange groups, provides researchers with a means of fractionating small-volume samples rapidly using the selective properties of ion-exchange membranes. Stated applications include the fractionation of proteins in serum, separation of proteins from nucleic acids, clean up of fluorescein-labelled oligonucleotides and the purification of enzymes. Researchers interested in larger-scale production of biomolecules can use the device to optimize separation conditions then scale-up directly to the company's MemSep HP DEAE rapid chromatography cartridges.

Gelman Sciences offers new, disposable 0.1 μm CritiCap-M **filter capsules** for the retention of Mycoplasma species when sterilizing large volumes of biological and tissue culture solutions (*Reader Service No. 112*). The company says that although industry standards still consider 0.2 μm filtration

devices to be acceptable, research would seem to indicate that *Mycoplasma* species can penetrate a membrane with a 0.2 μm pore size at relatively low pressure. The double-layer membrane capsule configuration utilizes the company's 0.1 μm Supor membrane.

Flexibility is the key with the new modular **DX500 ion chromatography system** from Dionex (*Reader Service No. 113*). A high-speed module-to-PC communications interface provides a unified system environment for real-time monitoring, intelligent diagnostics and digital data transfer. The DX500 is available with high-performance isocratic or quaternary gradient pumps. Flow rates can be varied from 0.01 to 10 ml min⁻¹. For compatibility with corrosive buffers and solvents, DX500 pumps are made from inert, polymeric PEEK-based materials. A selection of DX500 conductivity, electrochemical and optional detectors



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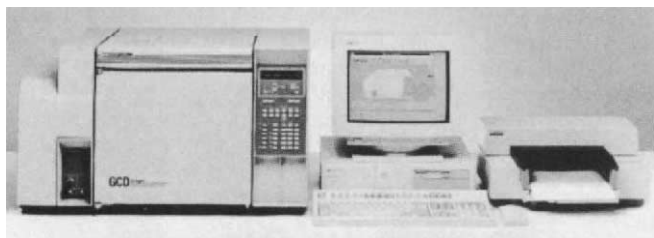
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Hewlett Packard's integrated GC detection system.

Hewlett-Packard's integrated **gas chromatography detector system**, the HP GCD system, is designed to provide chemists with qualitative structural information — in the form of an electron impact mass spectrum — with which to identify a broad range of chemical compounds (*Reader Service No. 114*). As well as the abundance and retention time, the mass spectrum provides a third dimension of data for each peak in a chromatogram. As most compounds possess a unique mass spectrum, HP says the system can be used to identify unknown peaks without the use of standards, including most compounds volatile below 400 °C and with a molecular weight below 425 atomic mass units.

Technical literature for the new PL-GPC110 high-temperature **gel permeation/size exclusion chromatography system** is now available from Polymer Laboratories (*Reader Service No. 115*). Entitled, *A Precision Thermostatted System for High Performance GPC/SEC*, the brochure covers topics such as sensitivity, reproducibility and system control. Technical information is provided on key elements of the system: oven, solvent delivery system, autosampler/injection system and the differential refractive index detector.

With the LiChrolut **solid-phase extraction system** from EM Separations up to 12 samples can be processed simultaneously (*Reader Service No. 116*). For nonpolar solid-phase extraction applications, LiChrolut SPE tubes are available in RP-select B, RP-18 and RP-18e. For biochromatography, LiChrolut tentacle ion exchangers are said to provide substantially higher capacity and recovery of biological activity. The system can be used to perform each step of the solid-phase extraction process, including conditioning of the cartridges, application of the sample, rinsing and washing, elution and recovery.

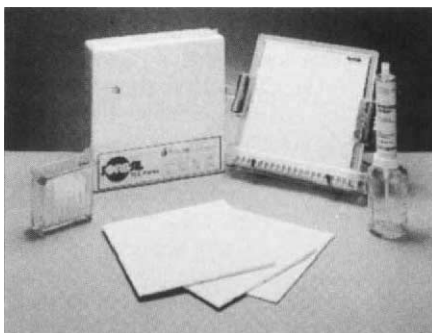
Columns

Econo-Column **chromatography columns** from Bio-Rad are designed for low pressure biomolecule purifications — ion exchange, gel filtration, affinity or hydrophobic interaction chromatography (*Reader Service No. 117*). Forty sizes of column are available, with lengths ranging from 5 cm to 170 cm, inside diameters from 0.5 cm to 5.0 cm, and total volumes of 1 ml to 1.3 l.

UltraLink R-PAC **prepacked protein affinity columns** from Pierce offer direct scale up in medium- and high-pressure chromatography systems (*Reader Service No. 118*). The support used in these new columns is the 3M Emphaze

biosupport medium AB 1. This has a large particle size of about 60 µm, and offers high capacity and high flow rates (3,000 cm h⁻¹). The columns are said to offer minimal nonspecific binding because the 3M Emphaze beads are very hydrophilic after coupling. The ligands are immobilized with a leak-resistant linkage to ensure minimal ligand leaching. The columns are made of borosilicate glass, which Pierce says has low adsorption properties to prevent the sample from sticking to the column.

Phase Sep's range of Sorbsil C silicas for preparative and flash chromatography has been extended with the recent introduction of **thin layer chromatography plates** (*Reader Service No. 119*). The plates, which

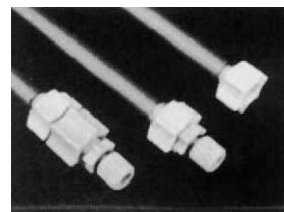


Sorbsil TLC plates.

incorporate a chemically and optically inert organic binder, should provide a useful tool for determining chromatographic solvent conditions prior to HPLC. Sorbsil silicas for flash and preparative chromatography include 30 Å and 60 Å irregular silica, both unbonded and cyanopropyl or C18

bonded. More specialized C18 derivatives designed for reduced peak tailing in applications directed more towards pharmaceutical research are also offered. Wide-pore Sorbsil material (unbonded and C18 bonded) is also available, offering high capacity and high throughput for larger molecules.

The new **wide-pore columns** from Upchurch Scientific use a highly purified gel designed specifically for chromatography of biomolecules (*Reader Service No. 120*). The company says



Upchurch Scientific's wide-pore columns.

Each batch of the 300 Å gel, which has a 90 m² g⁻¹ surface area, is selectively tested for resolution. In both the C4 and C18 phases, the surface is bonded with oligomeric phases, which are said to be stable to moderate (0.2 per cent) concentrations of trichloroacetic acid (TCA). The C18 phase is recommended for peptide separations, the C4, for proteins.

E. Merck now offers the **chromatographic sorbent**, hydroxylapatite, as bulk material of 15 µm particle size for preparative chromatography applications (*Reader Service No. 121*). The material is a spherical, porous carrier that can be used for the purification of enzymes, monoclonal antibodies, membrane proteins and nucleic acids. On request, the 15-µm sorbent is available as ready-to-use prepacked Super-performance glass columns. □

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